Post-Genomics Nanotechnology Is Gaining Momentum: Nanoproteomics and Applications in Life Sciences

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Abstract

The post-genomics era has brought about new Omics biotechnologies, such as proteomics and metabolomics, as well as their novel applications to personal genomics and the quantified self. These advances are now also catalyzing other and newer post-genomics innovations, leading to convergences between Omics and nanotechnology. In this work, we systematically contextualize and exemplify an emerging strand of post-genomics life sciences, namely, nanoproteomics and its applications in health and integrative biological systems. Nanotechnology has been utilized as a complementary component to revolutionize proteomics through different kinds of nanotechnology applications, including nanoporous structures, functionalized nanoparticles, quantum dots, and polymeric nanostructures. Those applications, though still in their infancy, have led to several highly sensitive diagnostics and new methods of drug delivery and targeted therapy for clinical use. The present article differs from previous analyses of nanoproteomics in that it offers an in-depth and comparative evaluation of the attendant biotechnology portfolio and their applications as seen through the lens of post-genomics life sciences and biomedicine. These include: (1) immunosensors for inflammatory, pathogenic, and autoimmune markers for infectious and autoimmune diseases, (2) amplified immunoassays for detection of cancer biomarkers, and (3) methods for targeted therapy and automatically adjusted drug delivery such as in experimental stroke and brain injury studies. As nanoproteomics becomes available both to the clinician at the bedside and the citizens who are increasingly interested in access to novel post-genomics diagnostics through initiatives such as the quantified self, we anticipate further breakthroughs in personalized and targeted medicine.

Introduction

THE POST-GENOMICS ERA HAS REALIZED that the sequenced genome is not enough to discern the global biological processes fully at a systems level (Collins et al., 2003; Gandhi and Wood, 2012). New “omics” fields characterized by data-intensive research and biotechnologies enabling omics investigation have come into existence to narrow the existing gaps between discovery science and the attendant clinical applications. One of the significant contributors in the post-genomics era is the field of proteomics. The rising interest in protein science is believed to be secondary to the far biological distance between genes and phenotypes on the one hand and

the dynamic nature of proteins on the other (Aebersold and Mann, 2003; Altelaar et al., 2012).

Chief among the aims of proteomics is the analysis of cellular proteins in terms of abundance and dynamics in response to physiological and pathological changes, as well as environmental influences. Proteins are central cellular components in biological networks, with diverse functions including cytoskeletal building blocks, enzymes catalyzing biochemical reactions, antibodies contributing to immunity, or transcription factors affecting gene expression. *Proteomics* by definition is the systematic identification and characterization of protein sequence, abundance, post-translational modifications, interactions, activity, subcellular localization,

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and structure in a given cell type at a particular time point (Zhang et al., 2013). Protein profiles at both physiological and pathophysiological processes characterize the information flow in a cell, tissue, or organism (Petricoin et al., 2002). Proteomics studies utilize several available techniques for the identification, validation, quantification, and expression of certain protein(s). Such techniques are highly sensitive achieving targeted proteins analysis; among these tools are: Western blotting, ELISA, and protein arrays, which are used for identification and quantification of proteins. On the other hand, proteomics can be of high throughput nature where a set of proteins are globally evaluated (expression and quantification) by methods including mass spectrometry, protein arrays and 1D and 2-D gel electrophoresis (Kobeissy et al., 2008b; Lamond et al., 2012; Smith and Figeys, 2006).

It is generally accepted that the human genome consists of around 40,000 genes (Lander et al., 2001; Yates, 2013), yet a single gene does not necessarily translate into one protein, and once proteins are synthesized, many undergo post-translational modification (PTM) by phosphates, carbohydrates, lipids, or other groups, which tremendously complicates the global proteome profiling (Mann and Jensen, 2003).

Similar to the Human Genome Project, a Human Proteome Project (Cottingham, 2008) was initiated by a group of scientists from the Human Proteome Organization (HUPO) and was launched at the 2011 World Congress of Proteomics in Geneva, Switzerland (Omenn, 2012). In this project, scientists have to deal with approximately more than 1,000,000 proteins, which can then be further complicated by several protein modifications. The time, effort, and money it takes for a protein to be fully identified, sequenced, validated, and structurally characterized impose a true challenge for researchers (Lemoine et al., 2012; Yau, 2013). Consequently, the very early hope of characterizing the whole human proteome shifted focus on trying to find molecular differences between one functional state of a biological proteome system to another aided by systems biology analysis, which certainly provided more precise comprehensive data of the proteome profile (Cox and Mann, 2007).

Challenges in Proteomics

The rapidly growing field of proteomics has excelled in several disciplines in biology, including injury, cancer, aging, and different neurological conditions, as well as psychiatric conditions including drug/substance abuse, schizophrenia, and depression (Abul-Husn and Devi, 2006; Becker et al., 2006; Becker, 2006; Choudhary and Grant, 2004; Cochran et al., 2003; Dean and Overall, 2007; Dumont et al., 2004). Proteomics is one of the fastest growing fields of biochemical sciences; a PubMed search reveals 287,021 articles published in the past 2 years containing the word "protein," compared to 156,200 articles using the term "gene" (January, 2011–April, 2013), which may also reflect a shift in genomics studies towards proteomics investigations. Interestingly, the field of proteomics is in a continual growth with the introduction of some more specialized disciplines and subdisciplines such as neuroproteomics, psychoproteomics and nanoproteomics (Kobeissy et al., 2008a; 2008c).

Proteomics analysis is more complicated than genomics due to a number of challenges occurring at different levels of protein post-translational modifications (Choudhary and

Grant, 2004; Zhao and Jensen, 2009). For example, on the level of brain proteome, it is estimated that there exist around 20,000 brain proteins that are differentially expressed in the various regions of the brain (Wang et al., 2005a). Furthermore, it is difficult to associate mRNA expression to the protein expression levels (Denslow et al., 2003; Freeman et al., 2005; Morrison et al., 2002; Wang et al., 2004). This is due to several factors, including "alternative splicing," which is highly common in brain tissue, generating several copies of highly related splices from a single gene (Hunnerkopf et al., 2007; Missler and Sudhof, 1998; Morrison et al., 2002; Wu and Maniatis, 1999). It is estimated that a single gene can generate up to 10 protein isoforms (Kim et al., 2004; Williams et al., 2004). Thus, knowing the gene sequence is not sufficient to predict the possible translation pattern of that specific protein. Currently, there are approximately 430 possible protein post-translational modifications (PTMs) that can contribute to this complexity (Khouri et al., 2011; Woodsmith et al., 2013).

Post-translational modifications are defined as integral "chemical modifications of proteins that have implications on new protein functions in response to a specific cellular condition such as activation, turnover, downregulation, conformation, and localization (Berretta and Moscato, 2010; Husi and Grant, 2001; Khouri et al., 2011; Morrison et al., 2002; Witze et al., 2007). Furthermore, the different expression levels of certain proteins leading to huge dynamic range difference hamper the analysis of low expression proteins (Hortin and Sviridov, 2010; Zubarev, 2013). For example, there is 0.5 pg/mL of IL-6 compared to 35 mg/mL of albumin present in the serum that exemplifies the dynamic range difference of some protein expression levels analyzed using traditional proteomics techniques including mass spectrometry, ELISA, Western blotting, and protein arrays (Anderson and Anderson, 2002). Therefore, many of the potential biomarkers have concentrations in the femtomolar range while being immersed in a complexity of other biological components with concentrations that span 12–15 orders of magnitude (Mitchell, 2010; Rifai et al., 2006).

Furthermore, some protein classes are notoriously very difficult to analyze due to their intrinsic characteristics. For instance, membrane proteins constitute almost 30% of the open reading frames in the sequenced genome (Bagos et al., 2004; Lai, 2013; Vuckovic et al., 2013); however, they are very hydrophobic and buried in the lipid bilayer and tend to precipitate in aqueous buffers; thus, are harder to isolate. In addition, the field of proteomics lacks a DNA-PCR-like technique, which brings about sensitivity problems, associated with low abundant proteins.

Role of Nanotechnologies in Proteomics

Nanotechnology is defined as the systematic study of a particular system at the nanometer scale (1–100 nm) (Nie et al., 2007; Vo-Dinh, 2005). Considering that average bond lengths range in the picometer range (74 picometer for H–H bond, 200 picometer for C–I bond); this is basically the limit of the metrics at which molecules cannot be further manipulated at the molecular level.

Nanotechnology opens up unique opportunities, not only for material science research, but also for biology, medicine, and many other disciplines by manipulating individual atoms and molecules in a specific way that can fit into a

certain application (Petros and DeSimone, 2010). So, what is the relationship between nanotechnology and proteomics and why will nanotechnology be beneficial for proteomics applications?

As mentioned previously, proteomics technology is challenged by several limitations (PTMs, dynamic range, biological complexities, etc.), which in turn makes it incapable of achieving some of its goals in elucidating protein changes unless it is coupled with other methods.

The attractive point of nanotechnology is that it can reduce these difficulties and therefore help to draw new information out of biological systems that otherwise would not be possible by using conventional techniques. The field of nanotechnology has been associated with several proteomics applications such as phosphoproteomics/metal oxide nanoparticles, nanostructure surfaces for protein separation, and analytical detection of biomarker proteins using arrays techniques (Leitner, 2010; Nelson et al., 2009; Northen et al., 2007; Rissin et al., 2010). This merging between nanotechnology and proteomics has generated *nanoproteomics*, which is defined as a discipline of science involving the application of proteomics techniques aided by nanotechnology to enhance probing and evaluating protein systems (Archakov, 2007). As discussed by Vo-Dinh and colleagues (2005), several basic cellular structures (proteins, polymers, carbohydrates, and lipids) are molecules with similar sizes to various nanostructures. The similarity between these biological nanosystems and nanostructures have important implications in designing and manufacturing of the next generation nano-assemblies (nanotechnology tool kits, lipid vesicles, and dendritic polymers) that may have important medical and biotechnological applications (Chen et al., 2013; Zhang et al., 2009).

In this report, we will summarize the recent nanotechnology applications that have been applied in different proteomics-related applications. We will discuss nanostructured surfaces, nanoporous particles, magnetic nanomaterials, gold nanoparticles, carbon-based nanomaterials, polymeric nanostructures, quantum dots technology, and finally clinical utility of nanoproteomics, along with their technology commercialization.

Nanostructured Surfaces

Conventionally, the size of typical structures is on the order of micrometers; however, technologies in the area of nanotechnology have enabled us to achieve surface structure in the range of nanometers. Nanostructured surfaces have attracted big attention owing to their unique physical, chemical, and structural properties, including enormously high surface area, quantum confinement, and interaction with light (Hoheisel et al., 2010; Parker and Townley, 2007; Tawfik et al., 2012; Vo-Dinh et al., 2005). Not surprisingly, nanoparticles have found widespread use in proteomics applications that can be summarized into three basic areas: (a) scaffold for protein biosensing, (b) sample purification and enrichment tool, and (c) substrate for mass spectrometry analysis (Luong-Van et al., 2013). These diverse uses enhance the efficiencies of several proteomics applications, including ELISA and mass spectrometry-related techniques, as will be discussed.

Protein biosensing changes the morphology of a surface from plain to a nanostructured form and alters the sensing properties of that particular material by (1) increasing the

surface area and, hence, increasing the available binding sites, and (2) enhancing the accessibility of the target to the surface-immobilized probe. This, in return, allows the detection of specific target(s) with higher sensitivity and faster kinetics. Kang et al. (2005) reported that protein arrays prepared by silica nanotube membranes significantly improved the signal-to-noise ratio compared to plain analogs. Similarly, Kim et al. (2010) also showed that three-dimensional surfaces provide higher aspect ratios, improving the immobilization capacity of the capturing probe 5-fold and enhancing its detection ability to almost 15-fold. Another example of nanostructured material is porous silicon that was first discovered by Bell Laboratories in 1960s; it has been used for many protein sensing applications (Jane et al., 2009). Unique fluorescence properties and tremendous surface areas of porous silicon (500–800 m²/g) made it an ideal candidate for protein sensors (Sailor, 2007). Ressine et al. (2007) used porous silicon as a scaffold for antibody arrays and obtained detection limits down to 1 picomolar for IgG and 20 picomolar for prostate-specific antigen (PSA) in clinical samples.

Hill et al. (2009) explored the curvature effect of spherical gold nanoparticles on the loading density of probes. They showed that the binding to the nanoparticle surface is similar to that of a planar structure when the diameter of the particle is greater than 60 nm. Kelley et al. further explored this concept by controlling the topography of gold microelectrodes at the nanostructure level. Neither the increase of surface area nor the probe density were found to be the dominant factor in improving on the sensitivity but the nanotopography introduced onto the microelectrodes (Bin et al., 2010; Das and Kelley, 2011). Using the optimized topography, ovarian cancer biomarker CA-125 was detected to the limit of 0.1 U/mL without the need to covalent label the target or the probe nor to resort to sandwich complexes techniques (Das and Kelley, 2011).

Another application of nanoparticles is femtoliter arrays. This technology has been developed in studying single enzyme molecules, detection of low abundance protein biomarkers in biological fluids, and single cell analysis (Malhotra et al., 2012; Rusling et al., 2013). Since the properties of single molecules significantly differ from the bulk solution, this method enabled detailed studies of single proteins and their kinetic properties, as illustrated by Gorris and Walt (2010). Also, detection of low femtomolar biomarker proteins was achieved utilizing such techniques (for reviews, see Gorris and Walt, 2010; LaFratta and Walt, 2008; Rissin and Walt, 2006). The principle of detection enhancement basically relies on Poisson Statistics, dictating that at very low concentration values, femtoliter size reaction chambers may contain either one or no molecules (Rissin et al., 2010). Recently, this technology has been coupled with classical enzyme-linked immunosorbent assay (ELISA). “Femtoliter arrays” platform, used for protein detection, has been called “single molecule arrays,” which can enhance ELISA assay sensitivity up to 68,000 times, reaching zeptomolar (10⁻²¹) detection limits that enable identification of markers specific to prostate cancer (Rissin et al., 2010).

Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) has been a workhorse in proteomics studies; since the early days of its discovery (Karas et al., 2000). However, the presence of the matrix often causes heterogeneous co-crystallization of the matrix and analytes,

resulting in significant background ion intensity in the low-mass range (<500 m/z). As a nanostructured alternative, Siuzdak and co-workers have developed desorption/ionization on silicon (DIOS) technique using nanoporous silicon substrates for detecting small peptides and metabolites without any mass interference due to matrix ions (Wei et al., 1999). In DIOS-MS, the analytes in solution are directly deposited on a nanoporous silicon surface without chemical matrix addition (Fig. 1). This approach provides simplified sample preparation, more uniform surfaces, and less background noise at masses below 600 Da, which is considered a huge advantage compared to regular MALDI analysis using standard sample spotting (Wei et al., 1999). Chemically-derivatized DIOS surfaces can analyze peptides down to 800 yoctomole (10^{-24} moles, corresponding to 480 molecules).

Conceptually very similar, a new mass spectrometry method called nanostructure initiator mass spectrometry (NIMS) has been introduced (Fig. 1). In the NIMS technique, perfluorinated silanes are trapped into nanostructures and evaporated upon irradiation using nitrogen laser that transfers the analyte into the gas phase in a very efficient way (Northen et al., 2007). NIMS, compared to DIOS, further improved the detection limit (700 yoctomole, 10^{-24} of verapamil) of mass spectrometry. It has been applied for the analysis of broad range of analytes such as single cells, metabolites, and peptides (Northen et al., 2007). It has also been applied in mass spectrometric imaging studies (Yanes et al., 2009). In a recent study by Gulbakan et al. (2010), anodic nanoporous alumina was used efficiently to make well-ordered nanowell array surfaces. In this study, small molecules and peptides (adenosine, bradykinin, tripeptide) were analyzed by changing the pore depth (10–50 nanometers). Significant improvements in ionization were observed by increasing pore depth

compared to plain analogs. It has shown that real biological samples (BSA digest, carnitine metabolites) can also be analyzed on those surfaces as a proof of concept.

Dupre et al. (2012) has applied a similar approach for high throughput identification of tryptic digests, which is the starting point of MS-based protein identification studies. They showed that tryptic peptides from cytochrome C, beta casein, BSA, and fibrinogen can be analyzed with femtomolar sensitivity and improved sequence coverage can be obtained. All these findings illustrate the advantage of using nanoarchitecture surfaces in proteomics-related applications.

Nanoporous Particles

Sample preparation is also a very integral part in proteomics analysis (Ottens, 2009; Ottens et al., 2006). Blood (serum and plasma), urine, saliva, tears, cell lysates, tissue lysates, and proximal fluids are used to study proteome changes applied on biomarker discovery. However, due to the inherent challenges in proteomics investigation (as previously discussed), the use of multi-dimensional sample separation techniques constitutes an inevitable and essential part in proteomics. Commonly, traditional 1-D and 2-D gel electrophoresis, high-pressure liquid chromatography, and capillary electrophoresis have been traditionally used for protein separation and sample fractionation (Ahmed, 2009; Wiśniewski JR, 2009).

As an alternative to classical sample preparation methods, Geho et al. (2006) used nanoporous silicon for fractionation of serum components prior to mass spectrometry analysis. Geho et al. prepared initially nanoporous silicon surfaces by electrochemically etching silicon wafers and then functionalized the surface with an aminopropyl group. When serum samples

FIG. 1. Preparation of NIMS chips for high sensitivity mass spectrometry Analysis: (Step 1) A low resistivity ($525 \mu\text{m} \pm 25 \mu\text{m}$) p-type (B doped) silicon wafer is cut into small pieces ($25 \text{ mm} \times 25 \text{ mm}$). (Step 2) The cut silicon chips are immersed into piranha solution to clean the surface and remove any surface impurities. (Step 3) Nanoporous silicon surface is obtained by anodic etching of the wafer in HF/MeOH. (Steps 4 and 5) Nanostructured silicon chips are washed with copious amounts of water and dried with UHP nitrogen and perfluorinated NIMS initiator BisF17 is spotted on it. (Steps 6 and 7) NIMS chips are heated in an oven at 100°C for 3–4 sec and excess initiator is blown off with UHP nitrogen. (Step 8) NIMS chip is mounted on a modified MALDI plate with double sided conductive tape. (Step 9) NIMS analysis is performed using a standard MALDI-MS instrument (Thingholm et al., 2006).

interact with these surfaces, unique MS profiles were obtained. This technique was a good demonstration of enrichment of labile and carrier-protein-bound molecules in biological samples. Similar to this work, Hu et al. (2009) used mesoporous silica chips for proteome fractionation based on enhanced sieving properties of these nanostructure surfaces. Hu et al. showed that by surfactant-functionalized mesoporous silica, low molecular weight peptides can be specifically isolated and fractionated from complex biological samples. After fractionation, the response of mass spectrometry greatly improved by achieving better sensitivity in detecting low molecular weight peptides (Hu et al., 2009). In another study, Finnskog et al. (2006) showed that highly improved sequence coverage for prostate-specific antigen (PSA) and human glandular kallikrein-2 protein can be achieved by trapping trypsin protein in a porous silicon nanovials with protein amounts as low as 8 fmol. The technique was not only sensitive but also very fast. The digestion was achieved within 30 sec, as opposed to conventional trypsin digestion protocols that usually take up to 16–24 h with low sample amounts (fmol levels). In a very recent work, Fan et al. utilized a nanoporous silica-based method to isolate low molecular weight peptides from high molecular weight proteins in serum biofluids of metastatic melanoma patients as molecular signatures. This was followed by proteomics identification (Fan et al., 2012b). MALDI mass spectrometry analysis and rigorous bio-statistical analysis led to the identification of 27 peptides that might be used as potential biomarkers in metastatic melanoma. Yin et al. (2012) used C8-modified graphene@mSiO₂ nanoconjugates for enriching endogenous peptides prior to mass spectrometry. These nanoconjugates provide a very huge surface area (632 m²/g) and excellent capturing properties that can fish out peptides from standard protein digests, as well as from real biological mixtures such as mouse brain tissue. The Ferrari group has reported a similar strategy for finding low molecular weight biomarkers for breast cancer samples (Fan et al., 2012a). Peptides from the serum of nude mice with MDA-MB-231 human breast cancer were isolated by nanoporous silica particles and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Protein signatures unique to different stages of cancer development were identified. Their approach and results reported in this study possess a significant potential for the discovery of proteomic biomarkers that may significantly enhance personalized medicine targeted at metastatic breast cancer.

In a conceptually very similar work, Tan et al. (2012) has used nanoporous silicon particles. They tested the efficacy of these nanoparticles for enriching the low molecular weight proteome of serum from colorectal cancer patients and found that patient samples can be clearly distinguished from control patients by statistical analysis.

Magnetic Nanomaterials

Protein expression, purification, and modification has been well established with the existing biotechnologies; however, methods for low abundant protein enrichment and separation are still challenging. Magnetic materials hold great promise. First of all, they have high surface/volume ratio that provides high surface area for coating/binding of different substrates. Second, several affinity tags (antibodies, aptamers, lectins,

and affibodies) can be introduced on their surface with relatively easy chemistries. A third and probably most important feature is that the separation can be easily performed with a simple magnet. Owing to these properties, paramagnetic particles have been widely applied for protein isolation shown in Figure 2.

A widely used method for such purification is nickel nitrilotriacetic acid (NTA/Ni²⁺)-based magnetic separation for histidine-tagged proteins (Gu et al., 2006). Pioneering work by Xu et al. has been applied in developing a general strategy using NTA-modified iron platinum (FePt) nanoparticles for separation of histidine-tagged proteins at concentrations as low as 0.5 pM (Xu et al., 2004a; 2004b). Lee et al. (2004) introduced bimetallic nanorods comprising of gold and nickel to fish out proteins magnetically using the similar Ni-NTA chemistry. In this work Lee et al. used Ni-Au nanorods to separate IgG antibodies. In another report, Shukoor et al. (2008) employed multifunctional copolymer functionalized superparamagnetic Fe₂O₃ nanoparticles for immobilizing and subsequently isolating His-tagged recombinant protein silicate from marine sponge *Suberites domuncula*. This highly versatile biomagnetic separation methodology also allows the re-use of the magnetic nanocrystals. Antibody-conjugated magnetic particles were also used to isolate biomarker proteins from plasma samples of cancer patients. Ranzoni et al. (2012) has synthesized PSA antibody conjugated nanoparticles and isolated PSA from plasma samples in conjunction with pulsed magnetic fields.

Chou et al. (2005) synthesized antibody-conjugated magnetic nanoparticles as a tool for isolating cancer proteins before MALDI mass spectrometric detection. C-reactive protein (CRP) and amyloid P component were efficiently isolated from unfractionated human plasma and sub-nanomolar level detection was achieved in MALDI analyses. In a follow-up study from the same group, serum amyloid A (SAA), C-reactive protein (CRP), and serum amyloid P (SAP) antigens were captured, isolated from human plasma, and detected by MALDI-MS in a multiplexed immunoassay format. In a recent study, Bamrungsap et al. (2011) used aptamer-conjugated superparamagnetic particles to detect lysozyme by using magnetic relaxation upon target capture.

Gold Nanoparticles

Another form of nanomaterials are gold (Au) nanoparticles that have been utilized for protein immobilization owing to their high affinity to thiol (-SH) and disulfide (S-S) groups present in various molecules. Their exceptional optical, scattering, and agglomeration-dispersion properties render them optimal candidates for various types of biolabeling applications (Kneipp et al., 1999; Rosi et al., 2004). Protein-mediated agglomeration of Au nanoparticles generates large local electromagnetic fields between immediate neighbors when illuminated known as “hot spots” (Lee et al., 2006). This technology enables researchers to analyze proteins within interparticle spaces using surface-enhanced Raman spectroscopy (SERS) reaching detection levels down to single molecule level (Gunnarsson et al., 2005; Kneipp et al., 1998; Kneipp et al., 1997; Nie and Emory, 1997; Podstawka et al., 2004; Wang et al., 2005b). Various colorimetric sensors for detecting metabolites, proteins, small molecules, and whole cells in solution as well as in real samples were developed by

FIG. 2. (A) Antibody-conjugated nanoparticles preparation for immunoassays: Antibodies are first tethered onto magnetic nanoparticles with a biocompatible conjugation chemistry (e.g., avidin-biotin, amine-carboxyl coupling, and click chemistry). A complex proteome (e.g., serum, plasma, cell lysate, saliva) is then incubated with the antibody-conjugated nanoparticles, and a magnet is utilized for magnetic separation. Finally, particles are washed and spotted onto sample plate and analyzed by MALDI-MS. (B) Enrichment of low molecular weight serum proteome: In the first step, serum proteome sample is incubated with silica nanoparticles and adsorbed or size excluded by mesopores on the silica NP, and the sample is centrifuged and NPs are separated. The nanoparticles are spotted onto sample plate and analyzed by MALDI-MS.

combining highly specific recognition properties of aptamers (single stranded target specific DNA molecules) and optical properties of Au nanoparticles (Pagba et al. 2010; Wang et al., 2010). These sensors are used to develop smart strip-type biosensors for *point-of-care* devices (Yoon, 2013).

In addition, gold nanoparticles play an important role in the field of quantitative nanoprotemics based on the barcode assay developed by Nam and colleagues (Georgiopoulou et al., 2005; Nam et al., 2003; Thaxton et al., 2009). This methodology is used for enzyme-free ultrasensitive detection of proteins and nucleic acid targets with sensitivity much higher than that of conventional ELISA-based assays. The same group also reported that PSA protein detection approached 330 fg/mL in the sera of patients who have undergone radical prostatectomy (Thaxton et al., 2009).

Gold nanoparticles can also be applied for the enhancement of biomarker discovery strategies through enriching low abundance proteins that were usually missed by conventional detection techniques. Yasun et al. (2012) used aptamers conjugated to gold nanorods for the detection of rare proteins, and demonstrated that these gold nanorods can be used to enrich proteins present at low abundance and increase the efficiency of their detection by 47%. The group showed that the used probes were able to detect thrombin in a buffer up to 10 ppb. Later, the same group was the first to test the use of hybrid Au-metal oxide nanomaterial as substrates for LDI-MS

(Ocsoy et al., 2013). They used Au@MnO nanoflower-shaped nanoparticle matrix for LDI-MS that was found to be an efficient nanoparticle substrate to LDI-MS as compared to other nanoparticles. The same nanoflowers were also used to target cancer cells and for the selective metabolite extraction and detection from cancer cell lysates.

Carbon-Based Materials

Elemental carbon in the sp^2 hybridization can form a variety of different structures. Among these, graphite, nanodiamond, carbon nanotubes, fullerene, grapheme, and graphene oxide, which are different forms of carbon nanostructures, have been well studied in different applications. Larsen et al. (2002) used graphite nanopowders in gel loading tips for desalting and preconcentration of peptides prior to mass spectrometric analysis, which is commonly used in MALDI sample preparation or other protein clean up settings. The same group showed that hydrophilic phosphopeptides can be significantly enriched using graphite powder (Larsen et al., 2004). Similar to that, Li et al. (2005b) used silicon-graphite hybrid coating for on target desalting. Among other forms of carbon, carbon nanotubes (CNTs) have also attracted great attention, since they have numerous novel and useful properties investigated since 1991. CNTs' length (up to several microns) with small diameter (a few nanometers) results

in a large aspect ratio. This makes CNTs very attractive material for proteomics applications via their wide surface area that enables better immobilization and capturing of proteins through functionalizing its surface with a capturing protein probe. For instance, Okuno et al. (2007) exploited the property of CNTs and designed a CNT-based electrochemical system to detect prostate-specific antigen from clinical samples. Furthermore, Chen et al. (2008) showed that a very sensitive Raman sensor that was able to detect low fM levels of protein biomarkers can be constructed by using CNTs.

In another study, Drouvalakis et al. (2008) designed a CNT sensor to detect autoantibodies in rheumatoid arthritis patients. In an elegant study, Guo et al. (2009) used carbon nanotubes to improve the resolution of native PAGE detecting serum proteins. Other proteomics application include: carbon based-nanostructures, which has also been applied as substrates for ionization in laser-based ionization techniques. Xu et al. (2003) used carbon nanotubes as a substrate for ionization. Sunner et al. (1995) used graphite for the same purpose. Hsu et al. (2010) used carbon nanotubes for screening long-chain fatty acids in patient samples. In another study, Najam-ul-Haq sputter coated nanostructured diamond like carbon on commercially available DVD disks and used this substrate for laser desorption ionization of peptides with a detection sensitivity of femtomolar levels (Najam-ul-Haq et al., 2008).

Graphene (G) and graphene oxide (GO) have been recently used in protein science. Tang et al. (2010) employed G & GO as a scaffold for enriching DNA and proteins in solution, yielding high recoveries which were four times higher than nanodiamond particles. Finally, Dong et al. (2010) used graphene as a substrate for ionization for the analysis of small peptides and small metabolites. Compared to conventional MALDI matrices, graphene exhibited higher desorption ionization efficiencies with very low background ions. Furthermore, it eliminated fragmentation and provided better tolerance to salts.

Carbon-based materials are also used in field effect transistors that allows for the real-time and fast detection of label-free proteins and biomolecules (Cui et al., 2001; Hahn Jong-in, 2004; Patolsky et al., 2004). A change in charge or electric potential at the nanomaterial surface is induced by the binding of a biomacromolecule. This in turn leads to a change in the charge carrier concentration in the semiconductor material that can be detected by conductometry measurements. Carbon nanotubes are relatively easy to prepare when compared to silicon nanowires and have a long shelf life (Tans et al., 1998). Chen et al. (2004) reported the detection of protein adsorption on single-walled carbon nanotube using field effect transistors (FETs). Furthermore, Chen et al. enhanced their work by modifying carbon nanotubes with DNA aptamers to recognize specific macromolecules selectively (Chen and Chen, 2005).

Polymeric Nanostructures

Polymeric materials, including polylysines, polyethyl-enimine, and cationic dendrimers, have been used in proteomics studies (Tao et al., 2005). However, the nano-architected nanomaterials turned out to be better alternatives to bulk polymers. Li et al. (2005a) used radiofrequency plasma polymers coated on a traditional MALDI plate for selective capture and detection of proteins that they termed as "on probe affinity capturing." The advantage of using this technique is that it allows the capture of different molecules by tuning the properties

of the polymers used (e.g., these can be thermo-responsive materials). For instance, N-isopropyl acrylamide is hydrophilic below a certain temperature and hydrophobic above that particular temperature. As a result, selective capture of hydrophilic or hydrophobic proteins can be achieved. Li et al. (2007a) showed that this thermo-responsive polymer coating in combination with temperature control can be used to clean up protein samples prior to mass spectrometry analysis.

Another interesting class of nanostructured polymeric material is the polymer brushes composed of a layer of polymers attached with one end to a surface (Jain et al., 2009). Polymeric nanostructures are also used as biomarker harvester; Luchini et al. (2008) used N-isopropylacrylamide nanogels in conjunction with affinity baits that functioned as molecular size sieve for capturing abundant proteins such as albumin. Longo et al. (2009) used the same nanogels to concentrate and preserve platelet-derived growth factor (PDGF) from serum in order to magnify the detectable level of the marker. This was performed in one single step and in solution phase (Longo et al., 2009) and has been commercialized under the trade name Nanotrap® (Shaffer, 2011).

Metal Oxide Nanoparticles and Phosphonoproteomics

Another area that has been addressed by nanotechnology and proteomics is the identification of PTMs and their structural characterization. More specifically, nanoparticles have been applied in characterization of protein phosphorylation, involving phosphopeptide isolation and subsequently identification by mass spectrometry as illustrated in Figure 3 (Olsen et al., 2010; Oppermann et al., 2009).

Protein phosphorylation is a ubiquitous and central process that regulates several different cellular functions such as signal transduction, cell growth and differentiation. Protein kinase activities are significantly elevated in different types of cancer and many new pharmaceuticals target phosphorylated proteins to help curb the disease progression. The state of the art method for identifying protein phosphorylation sites is mass spectrometry. However analysis and characterization of phosphoproteins from complex samples using MS is a real challenge. The ionization efficiency of cellular phosphoproteins is significantly hampered in mass spectrometry owing to their very low abundance relative to unphosphorylated proteins and to their negatively charged phosphate groups. Therefore, phosphoproteins are largely missed in high throughput proteomics experiments and phosphoproteomics has not been previously possible, due to the challenge of capturing and enrichment of low abundant phosphopeptides (Tao et al., 2005). The best way to address this problem is to use a capturing tool to pre-concentrate phosphopeptides prior to analysis by mass spectrometry (Engholm-Keller and Larsen, 2013; Johnson and Hunter, 2004).

The chemistry behind phosphopeptide enrichment remained immature until immobilized metal ion affinity chromatography (IMAC) was introduced (Feuerstein et al., 2005; Jin et al., 2004; Mann et al., 2002). The underlying principle of phosphopeptide enrichment is to form reversible and strong interaction between the metal and phosphorylation site of the protein. The task has been originally achieved by the use of IMAC resins that are mostly replaced with metal oxide-based affinity chromatography (MOAC) (Lin et al., 2008; Lin et al.,

FIG. 3. Phosphopeptide capture by metal oxide nanoparticles technology: (Step 1) Complex proteome sample (e.g., serum, plasma, cell lysate) is first digested with trypsin. (Step 2) Peptide mixture is incubated metal oxide nanoparticles. (Step 3) After incubation, metal nanoparticles are isolated from non-phosphopeptides and thoroughly washed. (Step 4) Phosphopeptides are eluted from the metal oxide nanoparticles and fractionated by SCX and RP-HPLC and infused into high resolution mass spectrometry. (Step 5) A survey scan is performed to identify most abundant peptides. (Step 6) Selected peptides are fragmented by collision induced dissociation (CID) and electron transfer dissociation (ETD). (Step 7) Phosphopeptides are identified by proper software (Johnson and Hunter, 2004).

2009; Thingholm et al., 2006; Zhou et al., 2007). Metal oxides behave as Lewis acids and show different acidity which forms the basis of targeted-specific selection observed in phosphoproteomics.

Phosphoproteomics was further supported by the introduction of enhanced mass spectrometry instruments and dissociation chemistry that facilitated the identification of different PTMs. For example, the use of high resolution mass spectrometry instruments and advanced electron transfer dissociation (ETD) analysis enabled the successful identification of these phosphopeptides (Coon et al., 2005). This was widely applied in identification of phosphorylation sites in embryonic stem cells (Swaney et al., 2009). Ultimately, profiling of a large number of different classes of phosphoproteins became possible and so emerged a (sub)-discipline termed as 'phosphonoproteomics' (Najam-ul-Haq et al., 2012).

Oxides of group 4 and 5 elements such as Ti, Hf, Zr, V, Nb, and Ta are the commonly used metal oxides (Najam-ul-Haq et al., 2012), among which titanium dioxide (TiO_2) is the favored metal oxide for phosphoprotein enrichment (Chen and Chen, 2005; Liang et al., 2006). Several different titanium dioxide-based nanostructures with various shapes and geometries have been used for phosphopeptide enrichment. For example Lu et al. (2010) have used TiO_2 nanocrystal clusters for enriching phosphopeptides. A mixture of b-casein, horseradish peroxidase, β -lactoglobulin, and fetuin was incubated with these nanoclusters for phosphopeptides enrichment. Torta et al. (2009) used pulsed laser deposition (PLD) to create nanostructured thin films on a MALDI plate and de-

veloped an on-plate enrichment protocol. This device, known as T-plate, unifies the sample enrichment and mass spectrometric analysis. He et al. (2011) used TiO_2 -coated ZnO nanorod arrays in a capillary microchannel to bind and enrich phosphopeptides selectively.

Several different nanocomposites of TiO_2 were additionally tested for phosphopeptide enrichment. As notable examples, Fang et al. (2012) used titanium dioxide-multiwalled carbon nanotube (TiO_2 -MWNT) nanocomposites, Zeng et al. (2012) used TiO_2 -coated carbon-encapsulated iron nanoparticles, and Lu et al. (2012) has used TiO_2 /graphene composites. Commercially, another form of functionalized TiO_2 is available composed of "TiO₂ coated magnetic nanoparticles." They represent a new hybrid class of phosphopeptide enrichment tool.

Another metal oxide used for enrichment is zirconium dioxide (ZrO_2), which was first introduced by Kweon and Hakansson (2008). Similarly, other functionalized ZrO_2 nanomaterials were used for phosphoprotein enrichment (Li et al., 2007a; Li et al., 2007b; Zhou et al., 2007).

Aluminum hydroxide (Al(OH)_3) (Chen and Chen, 2008) and iron oxide coated-Niobium oxide (Nb_2O_5) (Lin et al., 2009) are few other metal oxides that have been also used in selectively trapping phosphopeptides from peptide mixtures such as tryptic digest of caseins, serum, and cell lysate. Magnetic materials do show some degree of affinity to phosphopeptides (Lee et al., 2008) and have been majorly used as the core material for other peptide capture metal oxide (MO) coatings such as Fe_3O_4 @ TiO_2 (Chen and Chen, 2005; Li et al., 2008) or Fe_3O_4 @ ZrO_2 (Li et al., 2007a). This coating improves

purification and separation of target peptides upon selective binding from a complex peptide mixture, using external magnetic field.

When the performance of metal oxide nanoparticles were compared to that of conventional IMAC resins, it was found that metal oxide nanoparticles out-perform IMAC resins. Larsen et al. (2005) compared the performance of titanium oxide micro-columns with IMAC resins and found that significantly higher number of non-phosphorylated peptides were observed in the IMAC experiments. They reported that performance of the TiO₂ based-techniques significantly surpassed the IMAC method with respect to the number of detected phosphorylated peptides and reduction of the number of nonphosphorylated peptides. This is attributed to the more selective binding of phosphorylated peptides to TiO₂ micro-columns than IMAC resins.

Quantum Dots

Fluorescence-based techniques for protein sensing applications have gained an ever increasing popularity due to their simplicity and exquisite sensitivity (De et al., 2009; Ibraheem and Campbell, 2010; You et al., 2007). In particular, quantum dots (QDs), also called semiconductor nanoparticles, are emerging as a new class of fluorescent probes (Boeneman et al., 2009; Larson et al., 2003; Pinaud et al., 2006). When compared to organic dyes and fluorescent proteins, QDs have unique optical and electronic properties that make them brighter and highly resistive to photobleaching and chemical degradation (Leutwyler et al., 1996; Murray et al., 1993). The maximum emission depends on the size of the electron gap that is tuned by the particle core diameter. Smaller nanoparticles have a maximum emission in the blue region and bigger particles tend to emit in the red or near-IR.

These unique optical properties make QDs an excellent fluorescent probes for applications in the field of diagnosis (Bruchez et al., 1998; Chan and Nie, 1998), *in vivo* and *in vitro* imaging (Kim et al., 2003; Levene et al., 2004; Rosenthal et al., 2002), multicolor cell imaging (Hanaki et al., 2003; Jaiswal et al., 2002; Sukhanova et al., 2004; Wu et al., 2002), cell and protein tracking (Dahan et al., 2003; Voura et al., 2004), and DNA and protein sensing (Medintz et al., 2003; Zhang et al., 2005).

More recently, Liu et al. (2010) reported the detection and characterization of four low-abundant protein biomarkers (CD15, CD30, CD45, and Pax5) in Hodgkin's lymphoma using the multiplexing capabilities of QDs. QDs were also used to detect apolipoprotein E, the most important known genetic risk factor for Alzheimer disease, by designing a sandwich immunocomplex microarray assay based on cadmium-selenide/zinc-sulfide (CdSe@ZnS) quantum dots. The assay provided a low detection limit of 62 pg mL⁻¹, seven times more than that of the ELISA (470 pg mL⁻¹) when tested under the same conditions (Morales-Narváez et al., 2012).

A major drawback, however, to the utilization of QDs in the field of proteomics is the nonspecific binding onto their surface (Pathak et al., 2007). To minimize such nonspecific binding, QDs are often modified with polyethyleneglycol (PEG)-based polymers (discussed later) (Geho et al., 2005; Liu et al., 2008). More recently, Breus et al. (2009) capped QDs with small zwitterionic molecules. This step rendered the nanoparticles water soluble and dramatically diminished nonspecific binding.

Nanoproteomics in Clinical Applications

Nanoproteomics technology has been applied to various clinical settings, mainly for enhancing biomarker discovery capabilities. In this regard, nanoproteomics are seen to be functioning as protein amplification techniques similar to PCR. Potential clinical applications include infectious, endocrine, autoimmune, and neurodegenerative diseases, as well as brain injury and several types of tumors (Ray et al., 2011). Some of these applications are illustrated in Table 1 and Figure 4.

As applied to infectious diseases, Ansari et al. (2010), Kaushik et al. (2009a), and others used nano-structured surfaces for the detection of ochratoxin-A mycotoxin produced by *Aspergillus*. Ansari et al. utilized Nano-ZnO film deposited on an indium-tin-oxide (ITO) with immobilized rabbit-immunoglobulin and bovine serum albumin to reach a detection limit of 0.006 nM/dm of the toxin. Similarly, Kaushik et al. used nanostructured cerium oxide to achieve a detection limit of 0.25 ng/dL of ochratoxin (Kaushik et al., 2009a). Tang et al. (2004; 2005) proposed different immunosensors based on gold nanoparticles for the detection of hepatitis B surface antibody in blood. Reported detection limit of the applied techniques ranged between 5 and 15 ng/mL, which demonstrates a much higher sensitivity than the standard diagnostic serology techniques, allowing for better disease detection and monitoring. The same group also applied the immunosensors approach for the detection of serum levels of *Bacillus anthracis* protective antigen as well as HIV-capsid p24 antigen (Tang and Hewlett, 2010; Tang et al., 2009). These immunosensors approaches demonstrated rapid detection and much higher sensitivity compared to the traditional ELISA techniques that can reach up to 100 folds. Moreover, as part of a disease preventative measure, Yang et al. (2008) used carbon nanotubes for the detection of Staphylococcal Enterotoxin B in food products that were traditionally tested by ELISA. The new technique enhanced toxin detection limit and raised screening sensitivity by more than six-fold.

In the field of autoimmune diseases, many of the current diagnostic serology techniques do not demonstrate optimal sensitivity for diseases such as rheumatoid arthritis, celiac disease, Wegener's granulomatosis, and others. This could be in part due to the limited ability of these techniques to detect low levels of autoantibodies in the sera of suspected patients. Here, the application of nanoproteomics techniques could be promising. In fact, Drouvalakis et al. (2009) utilized peptide-coated nanotubes for the detection of rheumatoid arthritis-specific cyclic citrulline-containing peptide. The proposed technique was capable of detecting 12 out of 32 RA patients missed by ELISA and microarray (Drouvalakis et al., 2008). Several other studies have also reported the use of nanoproteomics for the detection of RA-related immunoglobulins (de Gracia Villa et al., 2011; Jiménez et al., 2012). Carbon nanotubes were also suggested as a screening tool for Wagner's granulomatosis (Chen et al., 2008). The authors used macromolecular single-walled carbon nanotubes (SWNTs) as multicolor Raman labels for multiplexed protein arrays for the detection of anti-proteinase 3 autoantibodies that are markers of Wagner's granulomatosis. Results have shown a three-fold increase in the detection level of these proteins compared to the recent fluorescent detection techniques. In addition, nanoproteomics techniques were also assessed for utilization in

TABLE 1. MAJOR STUDIES IN NANOTECHNOLOGY AND CLINICAL APPLICATIONS INCLUDING INFECTIOUS, ENDOCRINE, AUTOIMMUNE, AND NEURODEGENERATIVE DISORDERS

Study	Tool used	Disease/condition	Aim	Description
<i>Detection of bacteria/viruses/toxins</i> Nanostructured zinc oxide platform for mycotoxin detection (Ansari et al., 2010)	Nano-ZnO film deposited on an indium-tin-oxide (ITO)	Mycotoxin	Detection of ochratoxin-A	A detection limit of 0.006 nM/dm of the toxin was achieved
A novel immunosensor of hepatitis B surface antibody (Tang et al., 2004)	Tris(2,2'-bipyridyl)cobalt(III) and gold nanoparticles assembled on the PPF-modified Pt electrode	Hepatitis B	Detection of hepatitis B surface antibody	Comparable results to ELISA detection with a limit of 0.005–0.015 µg/mL
Detection of anthrax toxin by an ultrasensitive immunoassay using europium nanoparticles (Tang et al., 2009)	Europium nanoparticle-based immunoassay	Bacillus anthracis	Serum levels <i>Bacillus anthracis</i> protective antigen	The assay had a detection range of 0.01 to 100 ng/mL and was approximately 100-fold more sensitive
Carbon nanotubes based optical immunodetection of Staphylococcal Enterotoxin B (SEB) in food (Yang et al., 2008)	Carbon nano-tubes	Staphylococcal food poisoning	<i>Staphylococcal</i> Enterotoxin B in food products	The assay raised the screening sensitivity by more than 6-folds
<i>Detection of biomarkers for autoimmune diseases</i> Peptide-coated nanotube-based biosensor for the detection of disease-specific autoantibodies in human serum (Drouvalakis et al., 2008a)	Peptide-coated carbon nanotubes	Rheumatoid arthritis	Detection of rheumatoid arthritis-specific cyclic citrulline-containing peptide	The test was capable of detecting 12 out of 32 RA patients missed by ELISA and microarray.
Protein microarrays with carbon nanotubes as multicolor Raman labels (Chen et al., 2008a)	Macromolecular single-walled carbon nanotubes (SWNTs) as multicolor Raman labels for multiplexed protein arrays	Wagner's granulomatosis	Detection of anti-proteinase 3 autoantibodies	Three-fold increase in the detection level of these proteins compared to the recent fluorescent detection techniques
Celiac disease detection using a transglutaminase electrochemical immunosensor fabricated on nanohybrid screen-printed carbon electrodes (Neves et al., 2012)	Screen-printed carbon electrodes (SPCE) nanostructured with carbon nanotubes and gold nanoparticles	Celiac disease	Detection of IgA and IgG type anti-tTG autoantibodies	The proposed technique was comparable to ELISA
<i>Detection of cancer biomarkers</i> A voltammetric immunosensor based on nanobiocomposite materials for the determination of alpha-fetoprotein in serum (Giannetto et al., 2011)	Nanobiocomposite materials based on gold nanoparticles with immobilized antibodies	Hepatocellular carcinoma, germ cell tumors and others	Serum alpha-fetoprotein (AFP) detection	The assay had a detection limit of 3.7 ng/mL.

(continued)

TABLE 1. (CONTINUED)

<i>Study</i>	<i>Tool used</i>	<i>Disease/condition</i>	<i>Aim</i>	<i>Description</i>
Integrated microfluidic systems with an immunosensor modified with carbon nanotubes for detection of prostate specific antigen (PSA) in human serum samples (Panini et al., 2008)	Glassy carbon electrode modified with multiwall carbon nanotubes	Prostate cancer	Prostate specific antigen (PSA)	This technique was a quick detection technique with higher sensitivity than traditional ELISA.
Nanogold-enwrapped graphene nanocomposites as trace labels for sensitivity enhancement of electrochemical immunosensors in clinical immunoassays: Carcinoembryonic antigen as a model (Zhong et al., 2010)	Nanogold-enwrapped graphene nanocomposites	Colorectal cancer	Carcinoembryonic antigen (CEA)	A low detection limit of 0.01 ng/mL was demonstrated
In situ amplified electrochemical immunoassay for carcinoembryonic antigen using horseradish peroxidase-encapsulated nanogold hollow microspheres as labels (Tang and Ren, 2008)	Horseradish peroxidase-encapsulated nano-gold hollow microspheres	Colorectal cancer	Carcinoembryonic antigen (CEA)	The assay lowered the detection limit of CEA down to 1.5 pg/mL.
<i>Drug delivery and disease monitoring</i> A logical molecular circuit for programmable and autonomous regulation of protein activity using DNA aptamer-protein interactions (Han et al., 2012)	Logical circuit based on DNA aptamer-protein interactions	Different applications	Autonomous, self-sustained and programmable manipulation of protein activity in vitro	An example of application involves monitoring the levels and activity of thrombin in plasma and delivers an inhibitory anti-coagulant accordingly
Quantum-dot-conjugated graphene as a probe for simultaneous cancer-targeted fluorescent imaging, tracking and monitoring drug delivery (Chen et al., 2013)	Quantum-dot-conjugated graphene	Cancer chemotherapy	Targeted therapy with doxorubicin	The system can deliver the antineoplastic drug doxorubicin to cancer cells and monitor its release out of the cell allowing for a safer and more targeted therapy
Mesoporous silicon nanotechnology for cancer application (Bouamrani et al., 2009)	Multistage delivery systems using mesoporous silicon and protein platform chips with MS/MALDI	Targeted drug therapy		The protein platform helps detect protein changes that can allow disease monitoring in association with MS/MALDI and the multistage delivery system allows for targeted drug delivery.

FIG. 4. Current clinical applications of nanoparticles/proteomics. **(A)** Use of gold nanoparticles for high sensitivity detection of hepatitis B surface antigen (HBsAg). **(B)** Use of carbon nanotubes for the detection of anti-citrullinated peptide antibodies in rheumatoid arthritis. **(C)** Use of polymeric nanostructures for enrichment of low molecular weight proteins. **(D)** Use of quantum dots for detection of Apolipoprotein E detection in Alzheimer's disease. **(E)** Aptamer based circuit that monitors the levels and activity of thrombin in plasma and delivers an inhibitory anti-coagulant accordingly. **(F)** Use of PEGylated liposomes for drug delivery into the CNS across the blood brain barrier (BBB) for treatment of brain injury, including stroke and traumatic brain injury.

celiac disease through the detection of anti-gliadin auto-antibodies using different techniques where high sensitivity detection potentials were demonstrated (Neves et al., 2012; Ortiz et al., 2011).

An important application of nanoproteomics tools resides in cancer screening (Ji et al., 2010). Several plasma protein biomarkers have been associated with different types of cancer, yet their detection by current diagnostic and screening techniques is not satisfactorily sensitive. Therefore, nanoproteomics tools have been investigated for their possible screening potentials in these cases. For example, Giannetto et al. (2011) utilized nanobiocomposite materials based on gold nanoparticles with immobilized antibodies for the development of serum alpha-fetoprotein (AFP) immunosensors (Giannetto et al., 2011). Serum levels of alpha-feto protein are associated with various types of tumors including germ cell tumors, liver tumors, and others. Two other areas that show major potential for cancer nanoproteomics application are the detection of prostate-specific antigen (PSA) and carcinoembryonic antigen (CEA). Panini et al. (2008) used carbon nanotubes for the detection of PSA in serum. The utilization of glassy carbon electrode modified with multiwall carbon nanotubes provided a quick detection technique and a higher sensitivity than traditional ELISA. On the other hand, Zhong et al. (2010) employed highly sensitive electrochemical immunosensor with a sandwich-type immunoassay format to detect CEA that is associated with multiple tumors including colorectal cancer. This technique lowered the detection limit of CEA down to 0.01 ng/mL. Furthermore, Tang et al. (2008) utilized horseradish peroxidase-encapsulated nano-gold hollow microspheres as labels for the detection of CEA, lowering

the detection limit of this tumor marker down to 1.5 pg/mL (Tang and Ren, 2008). Several studies have reported the use of nanoproteomics as means for biomarker detection as in breast cancer, prostate cancer as well as others; for a detailed review, see Liu et al., (2013). Of interest, these nanoproteomic applications have been reported with different sensitivities ranging from 30 attoM to 2000 fMA even for the same antigen (PSA), however, using different assays as shown in Table 2.

A further detailed comparison among different biomarker detection sensitivities utilizing different nanomaterial-based approaches is presented in Table 3. In this table, HIV proteins, PSA, and Ochratoxin-A protein levels are compared and evaluated for their sensitivity detection limits using magnetic nanoparticles, metal oxides, composite nanosensor assay, Europium-based nanoparticles, carbon nanoparticles, gold nanoparticles, and porous silica coupled with mass spectrometry-based approaches.

Added to the value of nanoproteomics application in biomarker discovery, drug delivery, and personalized medicine are other fields where nanotechnology also holds future promise (Ferrari, 2005b; Kim et al., 2013). Nanotechnology-based "theranostics" applications were recently reviewed by Kim et al. (2013). Several applications for nanoparticle-based drug delivery have been proposed for therapeutics, including blood coagulation monitoring, cancer therapy, and stroke treatment; an eminent example of which is the use of liposomal nano-carriers (Alaoui and Sofou 2008). Han et al. (2012) described a logical circuit that enables autonomous, self-sustained, and programmable manipulation of protein activity *in vitro*. An example of such application is the use of a circuit that monitors the levels and activity of thrombin in

TABLE 2. COMPARISON OF DIFFERENT SENSITIVITIES AMONG CURRENT NANOTECHNOLOGY PLATFORMS FOR THE SAME PSA BIOMARKER

<i>Reference</i>	<i>Method</i>	<i>Detection limit</i>	<i>Biological fluid</i>
Nanoparticle-based bio-bar codes for the ultrasensitive detection of proteins (Nam et al., 2003)	Magnetic nanoparticle based bio-barcode assay	30 attoM	PSA detection in a background of goat serum
Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations (Rissin et al., 2010)	Femtoliter arrays	0.4 femtoM	Detection of PSA in the sera of patients who had undergone radical prostatectomy
Multiplexed electrical detection of cancer markers with nanowire sensor arrays (Zheng et al., 2005)	Nanowire sensor array	3 femtoM	PSA detection in undiluted serum
Nanoparticle-based bio-barcode assay redefines “undetectable” PSA and biochemical recurrence after radical prostatectomy (Thaxton et al., 2009)	Bio-barcode assay	8 femtoM	PSA detection in plasma after radical prostatectomy
A fluorophore-based bio-barcode amplification assay for proteins (Oh et al., 2006)	Fluorescent nanoparticle based Bio-barcode assay	30 femtoM	PSA in plasma
High-speed biomarker identification utilizing porous silicon nanovial arrays and MALDI-TOF mass spectrometry (Finnskog et al., 2006)	Silicon nanovial arrays	100 femtoM	Detection of PSA in human plasma
One-step homogeneous magnetic nanoparticle immunoassay for biomarker detection directly in blood plasma (Ranzoni et al., 2012)	Antibody-coated magnetic nanoparticles	400 femtoM	PSA detection in undiluted human blood plasma
Integrated microfluidic systems with an immunosensor modified with carbon nanotubes for detection of prostate specific antigen (PSA) in human serum samples (Panini et al., 2008)	Microfluidics carbon nanotube sensors	2000 femtoM	PSA detection in plasma

plasma and delivers an inhibitory anticoagulant accordingly. Chen et al. (2013), using a similar concept, designed a quantum-dot-conjugated graphene for imaging and monitoring drug delivery to cancer cells. The system can deliver the antineoplastic drug doxorubicin to cancer cells and monitor its release out of the cell, allowing for a safer and more targeted therapy. It is likely that this field of nanoproteomics

applications will be able to partake in the current quest for personalized medicine, coupling both disease monitoring and targeted therapy (Chen et al., 2013). In this regard, Bouamrani et al. (2009) proposed a multistage nanovector approach for both targeting and delivery of antineoplastic drugs. The system is built through combining multistage delivery systems: active components are carried by nanoporous silicon

TABLE 3. COMPARISON OF ANTIGEN DETECTION LIMIT USING DIFFERENT NANOPARTICLES/MASS SPECTROMETRY-BASED APPROACHES

<i>Technique utilized</i>	<i>Comparison of antigen detection limit</i>		
	<i>HIV proteins*</i>	<i>PSA*</i>	<i>Ochratoxin-A*</i>
Porous silica and mass spectrometry	NA*	8 femtoM	74 picoM
Carbon nanotube		15 picoM	24.1 nanoM
Gold nanoparictles	0.6 picoM	10 femtoM	20 nanoM
Europium-based nanoparticles	20 femtoM	NA	NA
Composite nanosensor assay	0.8 picoM	3 femtoM	NA
Metal oxides	NA	NA	6 picoM
Magnetic Nanoparticles	NA	30 attoM	NA
References	(Mahmoud et al., 2008; Tang and Hewlett, 2010)	(Finnskog et al., 2006; Nam et al., 2003; Thaxton et al., 2009; Zheng et al., 2005)	(Ansari et al., 2010; Guo et al., 2011; Kaushik et al., 2009; Yang et al., 2011)

*HIV proteins, PSA, and ochratoxin-A protein with their detection levels are evaluated using different nano-platforms including: magnetic nanoparticles, metal oxides, composite nanosensor assay, Europium-based nanoparticles, carbon nanoparticles, gold nanoparticles, porous silica, and mass spectrometry-based approaches (NA is Not Available).

particles. Then, microparticles are used to recognize “zip codes” on vascular endothelium, to detect environmental changes and allow extravasation of particles (Bouamrani et al., 2009).

Nanoparticles studies have been recently integrated in the area of neuroscience, particularly in the fields of stroke and brain injury, where the blood brain barrier (BBB) represents a major obstacle for drug delivery. Yun et al. (2013) generated nanoparticles that carry superoxide dismutase enzyme and targeted anti-NMDA (N-methyl-D-aspartate) receptor 1 (NR1) antibody. These nanoparticles were applied to a mouse model of cerebral ischemia where they limited reperfusion injury, and reduced apoptosis and inflammation, improving behavioral outcome. In addition, utilized coated PEG-based liposomes were also found to be localized to the CA region of the hippocampus, suggesting a probable mode of targeted delivery of reactive oxidative species quenchers in the treatment of stroke (Yun et al., 2013). Zhao et al. (2013) have tested Puerarin (PUE), a compound that suffers low availability in brain post-administration, as a candidate drug for treating stroke. In their work, PUE-loaded poly (butylcyanoacrylate) nanoparticles (PBCN) coated with polysorbate 80 (Ps 80), were tested for BBB crossing and effects on the cerebral ischemia/reperfusion injury. It was found that the vein injection of PUE-loaded PBCN exerted an improved neuroprotective effect in rats with focal cerebral ischemic injury, significantly decreasing neurological deficit and reducing the infarct volumes (Zhao et al., 2013). Similarly, Yin et al. (2012) used poly (n-butyl-2-cyanoacrylate) (PBCA) nanoparticles to deliver drugs for treatment of traumatic brain injury (TBI). The investigators tested the capacity of nanoparticles in crossing the blood brain barrier (BBB) and transporting large molecules into normal and injured brains. Of interest, at 4 h post-injury, the PBCA nanoparticle-delivered drugs showed a wide distribution near injured sites, indicative of an efficient delivery system for large molecules able to overcome the BBB observed in traumatic brain injury (Lin et al., 2012). Taken together, all these findings suggest that nanoparticles might be used as a delivery system to improve the transport of neurotherapeutic drugs to brain and have a potential as candidate neuroprotective agents in brain injury and stroke (Fig. 4).

Technology Commercialization

As has been reported in the previous sections, nanotechnology has made great contributions in the area of proteomics. Several of the shortcomings and limitations of proteomics studies were addressed. However, one aspect still to be discussed is the use of these technologies for the development of point-of care diagnostic devices, personalized medicine, and biomarker discovery.

While the future still hold great promise, to date, few technologies are used in clinical settings, and efforts have been largely restricted to academic research. One of the technologies currently in market is the BioBarcode technology (Hill and Mirkin, 2006). This is considered to be the most sensitive biological assay capable of detecting proteins and oligonucleotides in the attomolar concentration range. The assay is based on gold nanoparticles modified with a specific short oligonucleotide sequence and with an antibody that is specific to the target protein of interest. In a routine assay, the target is sandwiched between the magnetic and the gold na-

noparticles with a large ratio of bar-code DNA to target-protein. Upon the application of an external magnetic field, only the reacted gold nanoparticles from the reaction mixture are separated. In a subsequent step, the short oligonucleotides are released, amplified, identified, and quantified, allowing the magnification of the detection signal. This elegant sensing strategy is therefore extremely sensitive for protein detection. When compared to ELISA assays, the biobarcode assay (BCA) has been reported to be several orders of magnitude more sensitive, reaching low attomolar concentrations. Nam et al. (2003) reported a 30 attomolar concentration limit for the prostate-specific antigen (PSA). HIV-1 capsid protein (p24 antigen) were also detected with a detection limit of 0.1 picogram/mL, 150 fold more sensitive than conventional ELISA methods (Kim et al., 2008; Tang et al., 2007).

A major drawback to the BCA assay is the long time it requires to achieve a low detection limit. Indeed, it takes 3 days to execute all the steps; of that time, approximately 14 h is active and the rest is incubation time (Hill and Mirkin, 2006). Other versions of the assay have been reported with an assay time of 90 minutes but with a lower sensitivity (around 300 aM) (Oh et al., 2006).

The BCA is not solely a sensing assay but it is becoming an essential biological technique to understand diseases better by accessing low detection limits of specific markers. In the specific case of Alzheimer disease, for example, Georganopoulou et al. (2005) detected with attomolar sensitivity the concentration of amyloid- β -derived diffusible ligands (ADDLs). A significant difference was shown between concentrations of ADDL in Alzheimer's diagnosed subjects and in age-matched healthy controls, highlighting ADDL as a potential marker for Alzheimer disease.

Another technology that has made its way to clinical settings is CellSearch®. This technology detects circulating tumor cells in blood samples for early diagnosis of cancer. The technology relies upon incubating the blood sample with ferrofluids that consist of a magnetic core surrounded by a polymeric layer coated with EpCAM antibodies for capturing cells (Farace et al., 2011).

Finally, a new paradigm called P4 medicine was coined by Leroy Hood at the Institute for Systems Biology (Hood and Friend, 2011). P4 stands for predictive, preventive, personalized, and participatory medicine. Key to the success of P4 medicine is to detect the disease at an earlier stage, when it is easier and less expensive to treat effectively. This is where nanotechnology platforms would be greatly useful. NIH has initiated a program called “nanomedicine” in 2005 (Webster, 2006). The program aims at understanding the cellular biological machinery and its functionality at the nanoscale levels; data collected will be used to reconstruct cellular structures and help in initiating new projects and technologies that have translational dimensions in treatment and therapeutic potentials. Nanoproteomics is likely to become a big portion of these efforts, especially with the advent of the Common Fund's Protein Capture Reagents program introduced recently by NIH, as well as other affinity proteomics initiatives (Stoevesandt and Taussig, 2012).

Conclusion

The availability of nanotechnology materials and their applications in proteomics research has proved successful in

overcoming several technical limitations and road blocks associated with proteomics analysis including biological complexities, sensitivity, and dynamic range. These ultrasensitive and high-throughput technological platforms have opened doors to the rapid detection of low abundance proteins from complex and clinically relevant biological samples, facilitating identification and characterization of biomarkers for human diseases, potential candidates for clinical applications. Although this novel analytical approach has proved powerful, much more remains to be learned about the exact mechanism of action for the commonly used nanomaterials, and significant development is still required for incorporating such research into clinical care. Nevertheless, we anticipate an immense potential of nanoproteomics that may provide a greatly expanded approach to disease biomarker discovery, elucidating pathogenesis and mechanistic pathways in a variety of disease processes. In addition, these technologies might provide the possibility for early and accurate diagnosis, tailored drug selection, and monitoring of disease progression and therapy.

Despite that, a major challenge associated with the growing field of nanotechnology is increased human exposure to particulate matter and associated respiratory and cardiovascular toxicity (Kipen and Laskin, 2005; Medina et al., 2007). The smaller those nanoparticles go, the more reactive and thus toxic they are to the cellular environment. It is not only the use of these particles *in vivo* that will contribute to toxicity, as this is tightly regulated by FDA and other agencies (Ferrari, 2005a), but also the associated environmental pollution with these particles that will increase potential risks upon inhalation, especially among industrial workers (Donaldson et al., 2006). For instance, carbon nanotubes were found to be toxic to different cell types once tested *in vitro* such as keratinocytes (Monteiro-Riviere et al., 2005), T-lymphocytes (Bottini et al., 2006), and kidney cells (Cui et al., 2005). The toxicity of these particles is not only limited to the respiratory system, as they may evade the phagocytic mechanisms in the lung and leak into the systemic circulation and possibly the central nervous system (CNS) through various mechanisms (Medina et al., 2007). Eventually, this provides an avenue for nanoparticle-mediated neurotoxicity that may involve oxidative stress, inflammation, and apoptosis. Similar mechanisms are also thought to contribute to cardiovascular toxicity and atherosclerosis (Yamawaki and Iwai, 2006).

Therefore, despite the promises that nanotechnology holds for both basic science and clinical research, the escalating trend in using nanoparticles should be cautious and should take into concern environmental and toxicological "side effects." Eventually, this trend tries to fit within the toxicity-efficacy profile as any other drug or biological product. Several challenges face regulators in the field of nanotoxicology. These include the variability in behavior of the nanoparticles, bioavailability, neurotoxicity, biofluids solubility, and crossing blood brain barrier, which depends on several factors including size, encapsulation, and biochemical contents.

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