CHAPTER 7

Nanobiotechnology

SUMMARY

For the advancement of science, there is great interest in manipulation of molecules at the atomic level. The manipulation is of particular interest to electronic developers. Nanotechnology is using the structures of some cellular components as a starting point to construct nanoscale objects.

To visualize molecules and atoms at the nano level, scanning probe microscopes slide along the surface of an object and measure the magnetism, electrical resistance, temperature, or light resistance. From the data obtained, a raster image is displayed. The resolution of the probe depends on the size of it. There are two types of scanning probe microscopes: scanning tunneling microscopes (STM) and atomic force microscopes (AFM). The STM measures electrical resistance and also can move the atoms on the surface. Since the STM relies on electrical resistance, the surface being examined must be conductive. The AFM measures force between the probe and the sample. With the AFM, unlike the STM, the surface being examined does not have to be conductive. AFM can be used to detect the presence of viruses in a sample. In theory, numerous viruses may be detected from one droplet of blood using AFM technology.

There is immense potential in nanobiotechnology. For example, the weight of a single virus or bacteria can be determined using a nanoscale cantilever, similar to a diving board, and a laser then can detect the frequency of oscillation. Nanoparticles are constructed with various materials and in many different shapes. Some structures are spherical, flat, or rod-shaped, which could also be hollow or solid. The possibilities for the use of nanoparticles are immense. Fluorescent nanoparticles could be used to follow biological processes *in vivo*, such as the assembly and disassembly of protein scaffolds inside cells called microtubules. They are advantageous over traditional fluorescent labels for various reasons. They do not bleach out during exposure to UV light and therefore can be monitored for a longer period of time, and they are often brighter. Additionally, the wavelength admitted by the nanoparticle can be adjusted by altering the size of the fluorescent nanoparticle.

Nanoparticles can be used for the delivery of drugs or other biologically active molecules directly to a specific location. Proteins, DNA, or RNA could be delivered by nanoshells, which are hollow nanoparticles that can be packed with the molecules. Cancer therapeutic drugs could also be delivered directly to a tumor cell by a nanoparticle tagged with cell receptors specific to the cancerous cell.

Another application of nanobiotechnology includes the use of metallic antennas that coat double-stranded DNA molecules and cause the DNA to become single-stranded upon exposure to radio waves. Although the applications are futuristic, the possibility remains of controlling the switch between dsDNA and ssDNA inside living cells by administering a radio wave, which readily penetrates tissue.

Semiconductor nanocrystals can be synthesized by exposure of bacteria to specific compounds. For example, *Escherichia coli* precipitates 2 to 5 nm particles of cadmium sulfide when exposed to cadmium chloride and sodium sulfide. Fluorescent nanocrystals are produced when potassium tellurite is substituted for sodium sulfide. It is even possible for simple earthworms to generate fluorescent nanocrystals if given the correct ingredients. Nanocrystals can also be organized on the surface of bacteriophage, which are viruses that infect only bacteria. Interestingly, since some phage are filamentous, the nanocrystals formed on the surface of the phage become semicondutive nanowires. These nanowires could potentially be used as biosensors to detect the presence of a virus if they could be coated with antibodies against a specific virus particle.

Nanotubes are thin sheets of graphite that are rolled into cylinders of various diameters. They can act as semiconductors or conductors in electronics. Attaching enzymes, hormone receptors, or antibodies to nanotubes may provide a method to monitor a substance or

reaction through a nanosensor that detects an electrical signal. Nanotubes could also be stacked closely together to form a nanocarpet. Antibacterial nanocarpets have already been constructed from lipid molecules that act similar to detergents and degrade cell membranes. Surprisingly, the nanocarpets can also change colors depending on the presence of specific organisms or reagents. In the future, they could be used as a biosensor or to monitor bacterial contamination.

Target molecules could also be detected through the action of nanosensors. Nanosensors are ion channels embedded within membranes that control movement of molecules across the membrane. Antibodies that are attached to the channels serve as binding sites for molecules. Target molecules binding to the antibody could signal the ion channel to open or close, depending on the desired effect.

Nanoengineering of DNA uses the double helix as an intricate structural template that can be coated with a conductive metal for the creation of nanoscale circuitry. So far, only linear nanowires have been made by coating DNA with metals. Futuristically, it might be possible to generate 3D circuits from branched DNA structures or perhaps 3D structures that function as mechanical nanodevices. Such nanodevices could be used to perform a task by simply altering the base pairing between adjacent DNA strands.

A much simpler method for building nanostructures is through the computer-aided DNA origami approach. In this method, a very long DNA strand is folded up to form a scaffold. Other, shorter strands of DNA act as staples to help fold the larger strand. This method is much faster than traditional methods of building DNA nanostructures and the yield is higher.

The denaturation of DNA could also be controlled using nanoparticles. In this emerging technology, gold atoms are attached to DNA. During hybridization experiments, in which DNA needs to be denatured, the structure is exposed to radio waves from alternating magnetic currents. The gold atoms act as an antenna and are heated in a specific location. This denatures the DNA. Therefore, it is possible to selectively denature only those DNA molecules of interest to the researcher and may even have potential use in medicine.

Finally, molecular motors could one day power nanomachines and nanoassemblers by providing the energy source for the designated tasks. Currently, flagellar motor proteins, ATP synthase, and other complexes are being investigated to determine their feasibility in providing the energy to nanodevices.

Case Study Nanoproteomics: A New Sprout from Emerging Links between Nanotechnology and Proteomics

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Proteomics involves all the structures, modifications, and interactions of proteins within an organism at any one given time. The concept of proteomics is discussed more thoroughly in Chapter 9 within the textbook. In this review article, the authors discuss the idea of nanoproteomics, which is a bridge between the fast-growing genomics and nanotechnology fields and the slower-growing proteomics field. The authors identify two problems that plague the field of proteomics: limits in detection of proteins in biological samples and also the range of concentrations as a barrier to studying the proteins within a sample. Recently, proteomics has developed rapidly. Nanotechnology has also been applied to proteomics to yield a new field called nanoproteomics. Nanoproteomics has yielded technologies that are real-time, use less reagent and proteins, are more sensitive and robust, and produce results more quickly.

The field of nanoproteomics springboards from nanotechnology platforms by using carbon nanotubes, nanowires, quantum dots, and gold nanoparticles in proteomic research. In this review, the authors discuss the advantages and disadvantages of merging nanotechnology and proteomics.

How have nanomaterials been used in proteomic research?

Many different types of nanomaterials have been used in proteomic research. For example, organo-silica nanoparticles are used as packing material to increase separation of peptides during some chromatography applications. Gold nanoparticles are used in immobilizing and concentrating proteins from dilute samples. Gold nanoparticles also change colors when aggregated and can be used to elucidate protein–protein interactions. Carbon nanotubes are used as chemical sensors or electrochemical detectors, and have even been used to capture proteins in blood plasma. These nanotubes are able to capture traces of proteins from solutions. In general, nanoparticles are used for such proteomic activities involving identification, enrichment, immobilization, separation, and digestion of proteins.

Carbon nanotubules are hydrophobic. How has the hydrophobic nature of carbon nanotubules been overcome for use in aqueous solutions?

For any practical application in an aqueous solution, the surface of the carbon nanotubes must be modified either covalently or noncovalently. Covalent modification involves the addition of functional groups on the surface or sidewall of the nanotube and then linking the group with hydrophilic polymers or compounds. Noncovalent modification creates a carbon nanotube with a surface wrapped in surfactants.

Are there any advantages or disadvantages to any of the modifications discussed in the preceding question?

During covalent modifications to render the carbon nanotube functional in aqueous solution, the nanotube loses some intrinsic properties, including photoluminescence. However, using noncovalent modifications results in a surfactant-wrapped nanotube that does not lose any intrinsic properties. Consequently, many types of molecules can be anchored to the surface, including phospholipids and single-stranded DNA. The goal is to produce a functional carbon nanotube that is nontoxic, does not lose its coating, and has functional groups that can be used to conjugate other molecules such as antibodies.

What role do nanostructured surfaces play in protein identification techniques?

MALDI-MS is a protein identification technique that involves mixing peptides with an organic matrix and hitting the mixture with a laser. This results in ionization of the sample, followed by identification of the sample. During this process, the signatures for small peptides become overshadowed by the background signatures of the matrix and are difficult to identify. Nanostructured surfaces, such as silicon, metal oxides, and carbon nanotubes, provide suitable loading surfaces for the sample followed by identification of smaller peptides without the use of matrices.

Fluids contained in nanostructures behave differently then those in larger structures. Why does this occur, and how are these microfluids useful in nanoproteomics?

The behavior of nanofluids differs from their macro counterparts because of the scale of the fluid relative to their nanostructure containment. Nanofluidic filters have been created to far surpass conventional methods in protein purification and separation techniques. These filters are able to separate and fractionate protein and small DNA molecules in minutes.

Are there any medical uses for nanofluidic technologies?

Yes. An integrated blood bar chip uses nanofluidics with conjugated antibodies to bind, separate, and identify blood plasma proteins. Nanobiochips use a combined nanofluidic sensor with semiconductor nanoparticle to detect cancer markers in serum and saliva.

Nanoproteomics is the marriage of nanotechnology with proteomics. This combination has increased sensitivity and separation of peptides, enhanced detection for peptides, and decreased the use of large amounts of reagents and sample and research time. The authors believe this technology is trending toward an increase in nanoscale levels of separation and detection materials. Cumbersome conventional proteomics methods are being replaced with novel nanotechnologies, and the entire field is evolving at a fast pace.

Review

Nanoproteomics: a new sprout from emerging links between nanotechnology and proteomics

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The complexity of proteomics challenges current methods to provide all peptide mass fingerprints in an *ensemble* measurement of various proteins at differing concentrations. To detect those low-abundance proteins, nanotechnology provides a technical platform to improve biocompatibility, specificity, reproducibility, and robustness of the current proteomic methods. Here, we comprehensively analyze the weaknesses of traditional proteomic methods and evaluate the importance of nanomaterials in significantly improving the quality of proteomic methods by manipulating individual proteins. We also illustrate how the large surface-to-volume ratio of nanomaterials can facilitate mass transfer, enhance the efficiency of separation and high-throughput capability, and reduce assay time and sample consumption. The marriage of the two subjects and the resulting new nanoproteomics will revolutionize proteomics research.

The need for nanotechnology in proteomics

With completion of the human genome project in 2003, proteomics has become the point of interest for a deeper insight into the cellular processes that have not been depicted by genomics. The concept of proteomics involves a comprehensive study on the structures, localizations, post-translational modifications, functions, and interactions of all proteins expressed by an organism at a certain time and under certain conditions. Characterization of proteins will in turn lead to identifying the functional genome. Current estimates are that the proteome of eukaryotic cells contains thousands of proteins with up to 20 000 expressed at any time [1]. Such complexity poses a serious challenge to current proteomic techniques, including both the top-down 2D slab gel electrophoresis introduced 35 years ago [2,3] and the relatively new bottom-up approach using liquid chromatography/tandem mass spectrometry (LC/MS/MS) for peptide sequencing and mass fingerprinting (Box 1) [4,5]. In the 1990s, proteomics was not as strong as genomics. For example, proteomics missed tools like the polymerase chain reaction (PCR) in genomics, which can amplify a single nucleic acid molecule in a complex biological sample up to measurable levels that can be picked up by modern detectors [6]. Consequently, proteomics suffers from limit of detection (LOD): it is still impossible to detect all protein molecules existing in biological samples. Another problem in proteomics is the large dynamic concentration range of different proteins that co-exist in a single biosample – the so-called dynamic concentration barrier. Typical blood samples can contain more than 10 000 different protein species, with concentrations varying over nine orders of magnitude. Proteins at concentrations of 10^{-12}–10^{-15} M usually co-exist with those at concentrations of 10^{-3}–10^{-4} M, which makes detection of low-abundance proteins extremely difficult. Development of improved analytical techniques that can recognize a single protein molecule in the presence of others of high abundance is a top priority for proteomic methodology.

To face these proteomic challenges, protein microarray technologies have undergone rapid developments over the last decade, with cell-free expression systems now being employed for microarray generation [7]. Moreover, application of nanotechnologies to proteomics over the last few years has established a novel technical platform termed 'nanoproteomics' to study the dynamic concentration range of various proteins in complex biological samples. Advantages of nanotechnologies over conventional proteomic analysis include real-time multiplexed assays, low sample and reagent consumption, high sensitivity and specificity, short runtime, and miniaturized robust analytical systems composed of nanoscale separation media and channels. Unique nanomaterials such as carbon nanotubes (CNTs), nanowires, quantum dots (QDs), and gold nanoparticles (GNPs) are increasingly being used for proteomic applications. In this article, we briefly review conventional proteomic techniques and their weaknesses. We evaluate the benefits and risks of the merger between proteomics and nanotechnology and further analyze the scientific novelty, strength/weakness, and approaches, and discuss the perspectives of the technical marriage.

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Box 1. Shotgun sequencing and peptide mass fingerprinting

Shotgun sequencing is an approach used for analyzing a complex protein sample by LC/MS/MS, where the sample is digested with a suitable protease (commonly trypsin), and the resulting peptides are separated by HPLC and then characterized by tandem MS. Proteins are identified by matching the MS fragmentation patterns with predicted information from genomics or proteomics databases. Although many studies have used 1D reverse phase HPLC before MS measurement, such an approach is inherently limited by the number of peptides that can successfully be loaded and resolved on the column and detected by the mass spectrometer.

Peptide mass fingerprinting is a method for protein identification in which the unknown protein of interest is first cleaved into smaller peptides, whose absolute masses can be accurately measured with a mass spectrometer such as MALDI-TOF or electrospray ionization time of flight (ESI-TOF). These masses are then compared to either a database containing known protein sequences or even the genome by using computer programs to theoretically cut the protein into peptides, and calculate the absolute masses of the peptides from each protein. Then, the masses of the peptides of the unknown protein are compared to the theoretical peptide masses of each protein encoded in the genome. The results are statistically analyzed to find the best match. Therefore, the protein sequence has to be present in the database. In practice, each protein in a mixture has to be separated and isolated by 2D gels before cleavage.

Traditional proteomic strategies

Analysis of protein complexes based on a proteomics approach provides insight into specific translational signaling pathways and cross-interactions. Proteomics research takes these forms: (i) global proteomics to identify and catalogue all possible proteins within an organism [8]; (ii) targeted proteomics to identify and elucidate mutations and changes of individual proteins in complex mixtures of disease foci in the course of disease development [9,10]; and (iii) pharmacoproteomics to reveal interactions between proteins and drugs, and identify site-specific modifications (phosphorylation, glycosylation, and S-nitrosylation [11,12]) on the proteins induced by the drugs [13].

The first and perhaps the most crucial step in any successful proteomics experiment is sample preparation. Protein solubilization, protein separation, protease digestion, peptide separation, and peptide selection, all involve steps and protocols that vary greatly among laboratories. Moreover, different commercially available tandem mass spectrometers have different mass accuracies and different rates of peptide selection for fragmentation [14]. Proper sample preparation enhances the reproducibility of results. There is no one unique way to prepare samples and hence each protocol developed differs depending on the type of sample and the type of experiment to be conducted. Table 1 summarizes typical proteomic analysis techniques that are used to identify and quantify functional proteins present in the proteome.

Application of nanomaterials to proteomic analysis

Rapid detection of low-abundance proteomic samples requires the development of ultrasensitive, robust, and high-throughput technology. The above-mentioned techniques, however, are basically ineffective for direct identification of membrane proteins, insensitive for detection of low-abundance proteins, and impossible for assessing biological changes in protein regulation or expression [14]. In order to overcome these technical limitations associated with sensitivity, dynamic range, detection time, and multiplexing, proteomics has begun using nanotechnology resources resulting in a novel analytical platform known as nanoproteomics.

The employment of nanomaterials smaller than 100 nm in at least one dimension with their unique size-related physical and chemical properties into analytical technologies was first suggested almost 30 years ago [15]. However, their uses in proteomic analysis still lags behind the rapid development of other nanotechnologies, despite the numerous progresses made already [16,17]. New separation media feature higher efficiency and better selectivity in proteomic analysis than the traditional methods [18].

Table 1. Biotechnical advantages and disadvantages of current proteomic approaches

Technical categories		Advantages	Disadvantages	Refs
Non-mass spectrometry-based	2D gel electrophoresis	Can detect 1000s of proteins at a single run based on their size and charge	Narrow dynamic range; difficult for automation	[3]
	Multi-dimensional protein identification technology (MudPIT)	Strong cation exchange and reverse phase HPLC combined enhance sensitivity and dynamic range	Insensitive for low-abundance proteins	[62]
	Protein arrays	Surface is arrayed with specific molecules to the surface of the chip; easy for automation; good dynamic range	Require cloning of 100s of proteins	[7]
	Two-hybrid systems	Can detect potential protein interactions; easy for automation; measure DNA	False negatives and positives	[63]
	Isotope-coded affinity tagging	Broader dynamic range; easily automated	Measure relative abundance	[27]
Mass spectrometry-based	MALDI-TOF	Accurate mass measurement; fast and low cost	Extensive sample process prior to analysis	[13]
	Atmospheric pressure-MALDI	Fast and low cost, allowing for high-throughput analysis; good sensitivity	Extensive sample process prior to analysis	
	LC/MS/MS	Rely on fragmentation; broader dynamic range; reproducible; generate rich y, b, and neutral loss ions	Extensive sample process prior to analysis	[64]
	2D gel/MS	Simple sample process	Inefficient extraction of proteins from excised gel	[13]
	NanoMate	Faster and automated; favorable ionization; good sensitivity and reliability	High cost	

Table 2. Nanomaterials used in proteomic analysis

Nanomaterials	Function	Refs
Fullerenes	MALDI matrices for peptide and lipid analysis; peptide sequence identification	[43]
Iron-cored carbon ball (Fe–C)	Peptide sequence identification	
Carbon nanotubes	Peptides analysis; MALDI matrices; electronic sensor; sensitive immunodetection; nanobiocatalysis; affinity probes; peptides enrichment; protein digestion	[40,42–45,65]
Diamond nanoparticles	High-affinity capture of proteins for MS analysis	[66]
Amine-functionalized magnetic nanoparticles	Rapid and effective proteolysis	[67]
C8-functionalized magnetic nanoparticles	Enrichment of peptides in serum	[68]
Superparamagnetic maghemite nanoparticles	Carrier for trypsin immobilization	[69]
Trypsin-immobilized magnetic nanoparticles	Microwave-assisted digestion	[70]
Silica nanoparticles	Pseudostationary phase for protein separation; real time measurements and multiplex analysis	[71]
Polymeric nanoparticles	Protein adsorption	[72,73]
Copolymer nanoparticles	Identification of plasma proteins	[74]
$CaCO_3$-poly(methyl methacrylate) nanoparticles	Enrichment of low-abundance peptides	[75]
Silver nanoparticles	Antibacterial action against *Escherichia coli*	[76]
Gold nanoparticles	Enzyme immobilization; peptides and target-protein selection; high-throughput bioassay	[1,26,77]
TiO_2 nanocrystal	Selective enrichment of phosphopeptides	[78]
Zirconium dioxide	Specific enrichment of phosphopeptides	[79]
Ni/NiO core/shell nanoparticles	Selective binding and magnetic separation of histidine-tagged proteins	[80]

Nanomaterials have a large surface to volume ratio to facilitate mass transfer and increase efficiency of separation of various peptides [19]. For example, using organo-silica nanoparticles as the packing material in ultrahigh-pressure LC has achieved peptide separation speed 10 times faster than conventional methods without loss of high resolution [20]. The large surface area of nanomaterials can also be functionalized to facilitate specific separation of peptides. For example, monodispersed, superparamagnetic beads in nano sizes can be used as a support material to minimize sample loss and accelerate the processing speed via magnet-assisted separation [21]. Many nanomaterials show good chemical stability over a pH range much wider than silica (the most common packing material used in chromatography) [22]. Recently, nanomaterials have been employed for improvements of proteomic analysis especially when they are coupled with MALDI-TOF. Among the diverse classes of nanomaterials, gold nanoparticles, carbon nanotubes, silicon nanowires, and QDs are the most commonly-used nanomaterials to offer several advantages in nanoproteomics such as ultralow detection, short assay time, high-throughput capability, and low sample consumption. Table 2 summarizes the usefulness of these materials along with some promising materials such as magnetic nanoparticles, polymer, and metal oxide nanoparticles.

GNPs

GNPs are readily available, relatively stable, have low toxicity, and excellent compatibility with biomolecules [23]. GNPs can easily be modified with a large selection of functional motifs. Self-assembly monolayer technology increases GNP solubility and improves interfacial interactions. These functionalized GNPs can then be utilized in a variety of applications such as protein enrichment and enzyme immobilization [24]. Monodispersed GNPs are useful for concentrating proteins from a relatively large volume of dilute biological fluids by aggregation. This ability opens up new avenues of research because the traditional precipitation by trichloroacetic acid is ineffective in preparation of biosamples containing low concentrations of protein [24]. GNP aggregation is characterized by a red-to-blue color transition: the surface plasmon resonance band shifts. Thus, GNPs have also been widely used in high-throughput detection of biomolecular interactions [23,25].

Bottom-up proteomics analysis begins with protein digestion. Therefore, simple and reliable protocols for immobilization of enzymes are essential. The formation and activity of GNPs conjugated with different enzymes has been widely investigated. Enzyme molecules can bind tightly to gold colloidal particles and retain significant biocatalytic activity in a conjugated form when the enzyme molecules are denatured upon adsorption to planar surfaces of gold. Figure 1c shows the assembly of gold nanoparticles onto the zeolite surface through a linker of amine groups, resulting in a new class of biocatalysts with better activity, stability, and seven reuse cycles within a wide pH range (2–12) and temperatures (37–70 °C) [26].

Protein digestion always generates a very large number of peptides, which results in a great challenge to the downstream separation and MS identification procedures. One option to reduce the pool of peptides is to isolate the peptides that contain a specific amino acid residue. A porous polymer monolithic column covered with surface-bound GNPs exploits thiol chemistry for capture and separation of cysteine-containing peptides [1]. The condition

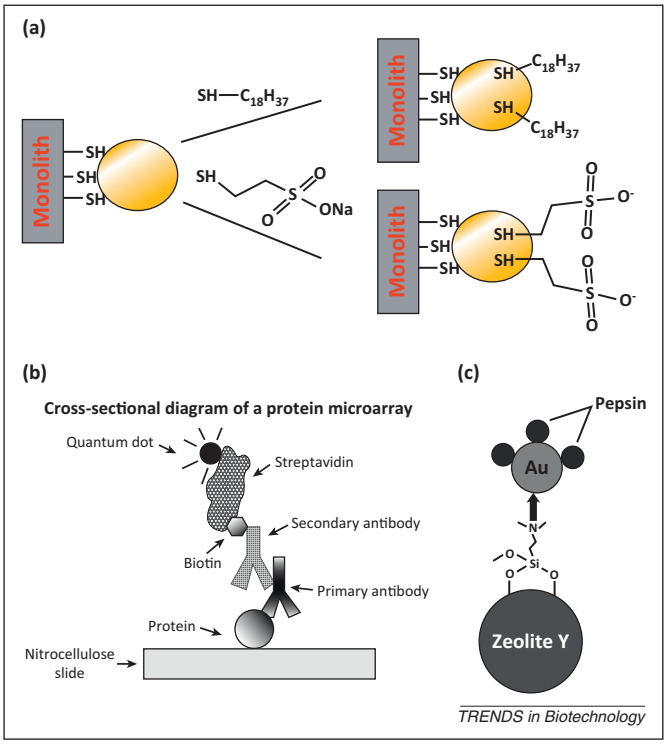

Figure 1. Examples of nanomaterials that aid in improving proteomic analysis. **(a)** Gold nanoparticles (GNPs; gold circles) conjugate thiols to develop a polymer monolithic column for capture and separation of SH-containing peptides [77]. The scheme shows that surface modifications of a poly(glycidyl methacrylate-co-ethylene dimethacrylate) monolith column containing GNPs (far left) with octadecylthiol ($SH–C_{18}H_{37}$) or sodium-2-mercaptoethanesulfonate results in a new column with exchangeable surface functionality. This process allows a switch between reverse phase (top right) and ion exchange (lower right) chromatographical separation of proteins. **(b)** Assembly of GNPs on the zeolite surface through the amine groups presented in 3-aminopropyltrimethoxysilane [48]. Pepsin binds the Na–Y zeolite (core)–Au nano (shell) structures via interaction with GNPs, leading to a new class of biocatalyst. This new biocatalyst is supported by a more massive biocompatible surface, and could easily be separated from the reaction medium by simple sedimentation. It exhibits excellent activity over seven successive reuse cycles as well as enhanced pH and temperature stability. **(c)** Reverse phase microarray used for signal amplification [26]. In the setting, proteins extracted from cellular lysates are arrayed onto a nitrocellulose substrate and probed with a primary antibody. In turn, a biotinylated secondary antibody recognizes the presence of the primary antibody. The biotinyl groups are then detected by streptavidin linked to reporter molecules such as quantum dots (QDs) or enzymes such as horseradish peroxidase that carry out catalyzed reporter deposition for signal amplification.

to release cysteine-containing peptides from the GNP column is different from that used for separation, making it possible for online coupling with LC/MS for capture, enrichment, release, separation, and identification of cysteine-containing peptides from non-cysteine containing peptides when separation of non-cysteine containing peptides precedes first. Based on this, a versatile monolithic GNP column was invented that allows easy switching between reverse and ion exchange separation modes using only one column (Figure 1a), which contains exchangeable surface functionalities to overcome the flaw in current existing methods that usually equip one column with single functionality [25,27,28].

CNTs

CNTs are another type of important nanomaterial useful for proteomic analysis. Since their discovery in 1991 [29], the cylinder-like materials have drawn intensive research interest because of their remarkable electrical, chemical, mechanical, and structural properties [30,31]. CNTs are classified as single-walled carbon nanotubes (SWCNTs) or multi-walled carbon nanotubes (MWCNTs). With diameters of 1–2 nm, and lengths ranging from 50 nm to 1 cm, SWCNTs are one-dimensional nanomaterials. They are amenable to biomedical applications because they have multiple binding sites for conjugation with targeting ligands, ultrahigh surface area (theoretically 1300 m^2/g): multiple molecules can be loaded along the length of the nanotube sidewall. The surface of nanotubes is polyaromatic so that aromatic molecules can be π–π stacked all over the nanotube.

The surface of CNTs is highly hydrophobic. For biomedical utilization, the CNT surface needs to be either covalently or noncovalently modified [32]. Covalent functionalization forms conjugation between carboxyl groups and functional moieties on the CNTs sidewalls, or cycloaddition onto the sidewalls. By contrast, noncovalent functionalization builds interactions between the hydrophobic domain of an amphiphilic molecule and the CNT surface to afford aqueous CNTs wrapped by surfactants. The two methods are briefly described in Box 2.

The unique properties of CNTs make them extremely attractive in designing chemical sensors in general and electrochemical detectors in particular. The work had been widely reviewed [33–37]. With increasing complexity in

Box 2. Covalent and noncovalent modification of CNTs

Covalent reactions [41] include (i) oxidation using oxidizing agents such as nitric acid (HNO_3) to form carboxyl groups at the ends of tubes or at the defects on the sidewalls, and then hydrophilic polymers (e.g., PEG) or other functional moieties (e.g., R–NH_2, R–OH, $SOCl_2$, or carbodimide) can be conjugated with the carboxyl groups; (ii) cycloaddition of aromatic sidewalls of CNTs with azides (R–N=N+=N–) under photochemical reaction; (iii) Bingel reaction between CNTs and carbine generating compounds [e.g., $Br(COOR)_2$] under a condition of strong base; and (iv) 1,3-dipolar cycloaddition on CNTs under dimethylformamide at 130 °C. Functional groups (e.g., amino-terminated PEG) introduced via the covalent reaction can be further conjugated with peptides and drugs. Disadvantages of the covalent functionalization include disruption of the intrinsic physical properties of CNTs resulting in losses of their photoluminescence and Raman scattering.

Noncovalent functionalization [40] of CNTs by coating their surface with amphiphilic surfactants or polymers essentially preserves the physical properties (e.g., π-network of CNTs) of CNTs. Aromatic molecules such as pyrene can bind to the polyaromatic graphitic surface of the CNTs via π–π interaction. Therefore, amine-reactive pyrene derivative, or glycodendrimer-conjugated pyrene can be anchored on to the CNT surface via π–π stacking. An SWNT coated with a single-stranded DNA or functionalized with PEGylated phospholipids has been made. Various amphiphiles have been used to suspend CNTs in aqueous solutions with hydrophobic domains attached to the CNT surface via van der Waals forces and hydrophobic effects, and polar heads for water solubility. Various surfactants such as Tween-20, pluronic tri-block, sodium dodecyl sulfate (SDS), and Triton X-100 copolymer have been tried to noncovalently functionalize nanotubes surfaces to reduce the non-specific binding of proteins and solubilize CNTs in water. The amounts of surfactants have to be carefully calculated to stabilize the CNTs while avoiding lysing of cell membranes and denaturation of proteins. The ideal noncovalent functionalization coating should: (i) be biocompatible and nontoxic; (ii) be sufficiently stable to resist detachment from the nanotube surface in serum; and (iii) have functional groups for conjugation with antibodies or other molecules.

systemic analysis and data integration, CNTs are showing greater potential as new tools in proteomic analysis [38].

SWCNTs are formed when one single layer of graphite is folded onto itself and the resulting edge is joined, with high aspect ratio, lengths from several hundred nanometers to several micrometers, and diameters of 0.4–2 nm [39]. A lot of effort has been put into both covalent and noncovalent functionalization of SWCNTs to overcome a major technical barrier: poor solubility in aqueous media [32,40,41]. SWCNT surface modification not only results in better dispersion but also provides better selectivity for bioanalysis and separation purposes. SWCNTs have been incorporated into an organic polymer monolithic stationary phase to separate peptides by capillary electrochromatography [42]. Pretreated SWCNTs dispersed in 2-propanol have been used as a porogen in the preparation of a monolithic column by employing vinylbenzyl chloride as monomer and ethylene glycol dimethacrylate as cross-linker. Both the retention factors and the separation efficiency on the SWCNTs monolithic column seemed to be quantifiably superior to those obtained from the control column without incorporated SWCNTs [42]. Functionalized SWCNTs have been used as MALDI matrices for peptides analysis [43]. Attachment of multiple acidic $(CH_2)_2COOH$ groups to the wall is critical for the matrix function. CNTs with functional groups attached to the nanotube ends cannot perform as MALDI matrices, nor do they work when the COOH end groups and multiple sidewall fluorine substituents are used. An important advantage of using SWCTNs is the absence of a low-mass matrix signal. This is in part due to the large size and relative stability of single nanotubes [43]. In addition, SWCNTs may have some advantages in protein analysis because of their larger size and payload.

MWCNTs are made of several layers of graphene cylinders that are concentrically nested like rings of a tree trunk with an interlayer spacing of 3.4 Å [39]. Although interactions between the layers still need to be further investigated to understand the accurate properties of MWCNTs [38], MWCNTs have already proven useful in proteomic analysis. They have been used as the assisting matrix for MALDI analysis of bradykinin, insulin, and cytochrome C without suppressing the low-mass ions in the presence of the high concentration of citrate buffer (50 mM) [44]. Thus, the mass detection range could be extended to 12 kDa and alkali metal adducts of analytes could be effectively reduced. Furthermore, citric acid-treated MWCNTs have been employed as affinity probes to selectively concentrate traces of analytes from aqueous solutions in the presence of high concentrations of salts and surfactants, which would otherwise have been intolerable. This approach is very suitable for the MALDI analysis of small proteins, peptides, and enzyme-digested products. MWCNTs have been used as an alternative adsorbent to capture endogenous peptides from human plasma peptidome analysis [45]. MWCNTs improved sensitivity and resolution of LC/MS. Twenty-five hundred and twenty-one peptide features (m/z 300–1800 range) could be detected in about 50 μL of plasma. Three-hundred and seventy-four unique peptides were identified with high confidence by 2D LC system coupled to a nanospray ionization linear ion trap MS. High recovery of BSA digest peptides enriched with MWCNTs, in both standard buffer and high-abundance protein solution, was observed. MWCNTs are superior to chromatographic columns of octadecyl carbon chain (C18)-bonded or C8-bonded silica in capturing the smaller peptides.

Nanofabricated structures used in proteomic analysis

Nanofabrication is the design and manufacture of devices with dimensions measured in nanometers. Here, only nanostructured surface and nanofluidics will be discussed regarding their applications to proteomic analysis.

Nanomaterials: nanostructured surface

MALDI-MS has been widely employed for protein identification using suitable organic matrices. Unfortunately, matrix peaks interfere with the fingerprints of small peptides whose mass-to-charge ratio is less than 800 m/z. Several kinds of metal and metal oxide nanoparticles as well as CNTs have been used to solve this problem [46]. One method uses porous silicon to trap analytes deposited on a porous silicon surface. Applied laser irradiation vaporizes and ionizes the biomolecules. The method works at femtomole and attomole levels of analyte, and induces little or no fragmentation, in contrast to what is typically observed with other approaches. The ability to perform these measurements without a matrix also makes it more amenable to small-molecule analysis. In addition, chemical and structural modification of porous silicon has enabled optimization of the ionization of the porous silicon surface. This technique is sensitive and compatible with silicon-based microfluidics and microchip technologies. Based on this, nanoporous surfaces have been used to capture low-molecular weight peptides from human plasma [47]. Here, the surface was fabricated by coating silicon chips with a 500-nm thick nanoporous film of silicon oxide. The average pore size was estimated to be about 7 nm. Harvested peptides were analyzed by MALDI-TOF MS and 70 peaks were found in the 800–10 000 m/z range. This method was able to detect peptides at the ng/ml concentration level [47].

Reverse phase protein microarrays have been used for proteomic analysis after the microarray surface is nanostructured. These nanostructured arrays facilitate high-throughput screening of post-translational modifications of signaling proteins from diseased cells. QDs have been used as reporter agents for amplification of the microarray sensitivity based on their multiplexing potential [48]. As shown in Figure 1b, the Qdot 655 Sav (an inorganic fluorescent nanoparticle conjugated to streptavidin) is used in a reverse phase protein microarray format for signal pathway profiling. Moreover, after pegylation, the pegylated Qdot 655 Sav bioconjugate has superior sensitivity and specificity to a non-pegylated form of the bioconjugate in terms of cellular protein extracts. Hyperspectral imaging of the QD microarray enabled unamplified detection of signaling proteins within defined cellular lysate, and thus this approach may be amenable to multiplexed, high-throughput reverse phase protein microarrays in which numerous analytes could be measured in parallel within a single spot.

Nanofluidics

Nanofluidics is the study of behavior, manipulation, and control of fluids that are confined to structures at nanometer level. Fluids confined in these structures exhibit physical behaviors different from those in larger structures because the physical scaling lengths of the fluid very closely coincide with the dimensions of the nanostructure itself. With advances in nanofabrication, it is now possible to reliably fabricate nanofluidic gaps and filters that have regular, controllable structures, with near-molecular dimensions (10–100 nm). One can take advantage of these nanofluidic structures for advanced separation and manipulation of various biomolecules and bioparticles.

Compared to conventional separation methods such as those using gels with random nanopores, nanofluidic sieves and filters have demonstrated superior performance for both analytical and preparative separation of various biomolecules including proteins. A microfabricated nanofilter array chip can size-fractionate SDS–protein complexes and small DNA molecules based on the Ogston sieving mechanism [49]. Nanofilter arrays with a gap size of 40–180 nm have been fabricated and characterized. Complete separation of SDS–protein complexes and small DNA molecules has been achieved in several minutes with a separation length of 5 mm. The fabrication strategy for the nanofilter array chip allows further increase in the nanofilter density and decrease in the nanofilter gap size. As a result, faster separation could be possible. A facile microfluidic colloidal self-assembly strategy can create orderly, robust, 3D nanofluidic sieves within microfluidic devices to achieve fast separation of DNA and proteins of a wide size range [50]. Compared to conventional colloidal deposition procedures such as vertical deposition, this approach features much faster assembling speed, absence of drying-caused cracks that may otherwise jeopardize the separation performance, and better flexibility to couple with current microfabrication techniques.

An integrated blood bar code chip consisting of a nanofluidic system having channels with immobilized antibodies can be used for on-chip blood separation and rapid determination of a large panel of protein markers over a broad concentration range. Nanofluidics can be efficiently combined with other sensing platforms rapidly detect low-abundance analytes. Nanobiochips, which consist of semiconductor nanoparticle QDs integrated into a modular microfluidic biosensor, have been used for detection of cancer markers (CEA, CA-125, and Her-2/Neu) in human serum and saliva samples [51]. Another unique application of microfluidics is for printing protein microarrays, which has until now been carried out by traditional pin and ink jet printing technologies that have limitations for printing protein solutions; the protein of interest is usually not present at a concentration high or pure enough for direct printing. A 3D microfluidic system is now devised to print protein samples in continuous flow to overcome concentration related drawbacks of other printing technologies [52,53]. This continuous flow microspotter could generate protein arrays from dilute samples at concentrations as low as 0.4 μg/ml with excellent spot morphology, uniformity, and increased surface concentration.

An anisotropic nanofilter array (ANA) was recently created for continuous flow protein size separation, including native proteins, SDS-coated proteins, long DNA, and short DNA, by utilizing different sieving mechanisms (Figure 2a–c) [54]. Structural anisotropy is the key to high-throughput, continuous flow separation, and also ideally suited for preventing the system from clogging, because larger molecules are prevented from entering many nanofilters [55]. The ANA structures have been used for a rapid and quantitative immunoseparation assay of proteins [54]. The ANA structures, composed of periodically patterned deep channels and shallow regions, allow small antibodies to pass through the shallow regions easier than large immunocomplexes, when the flow field is applied in an oblique direction. Human C-reactive protein and human chorionic gonadotropin can be examined by using fluorescent-labeled polyclonal antibodies. It has been shown that the size of the immunocomplex and the field strength are critical for separation [56]. Additionally, the device allows a convenient measurement of homogeneous binding kinetics without the need for repeating the binding experiments and immobilizing molecules.

Nanofluidics could also be used as a preconcentrator to enhance the detection limit of biomolecules, and concentrate proteins and peptides up to a million fold within 30 min (Figure 2d, e) [57]. Due to the electrical double layer overlapping determined by a sharp increase in fluorescence intensity, sub-100 nm nanochannels have preferential transfer over counterions (or counterion current). As a result, a well known phenomenon called ion concentration polarization can be observed. By coupling a tangential field across the ion depletion zone, fast accumulation of charged molecules can be achieved. The device recognizes the charged biomolecules based on two features: (i) the energy barrier for charged biomolecules generated by the induced space charge layer near the nanofluidic filter; (ii) a faster nonlinear electroosmotic flow for sample deliveries. The preconcentration factors (10^6–10^8-fold) and collection speed (a 10^7-fold concentration increase within 40 min), which are rate-limiting for many genomics researchers, are close to those of PCR for nucleic acids.

Almost all immunobiosensors are inherently limited by the quality of antibodies available for the target molecule. Consequently, obtaining a highly specific antibody for a given target molecule could be a challenge. A nanofluidic device integrated with charge-based electrokinetic preconcentrator with a bead-based immunoassay has been used as an immunoassay and to measure binding kinetics [58]. With a 30-min preconcentration, immunoassay sensitivity could be enhanced by more than 500 folds. Moreover, by adjusting the preconcentration time, a broader dynamic range (0.01–100 000 ng/ml) of detection could be obtained for a given bead-based assay. As the system can enhance both detection sensitivity and dynamic range, it can be used to address the most critical detection issues in the detection of common disease biomarkers.

Recently, a nanofluidic proteomic immunoassay was developed to quantify total and low-abundance protein isoforms in nanoliter volumes [59]. The method can quantify amounts of MYC oncoprotein and B cell lymphoma protein-2 (BCl_2) in Burkitt's and follicular lymphoma;

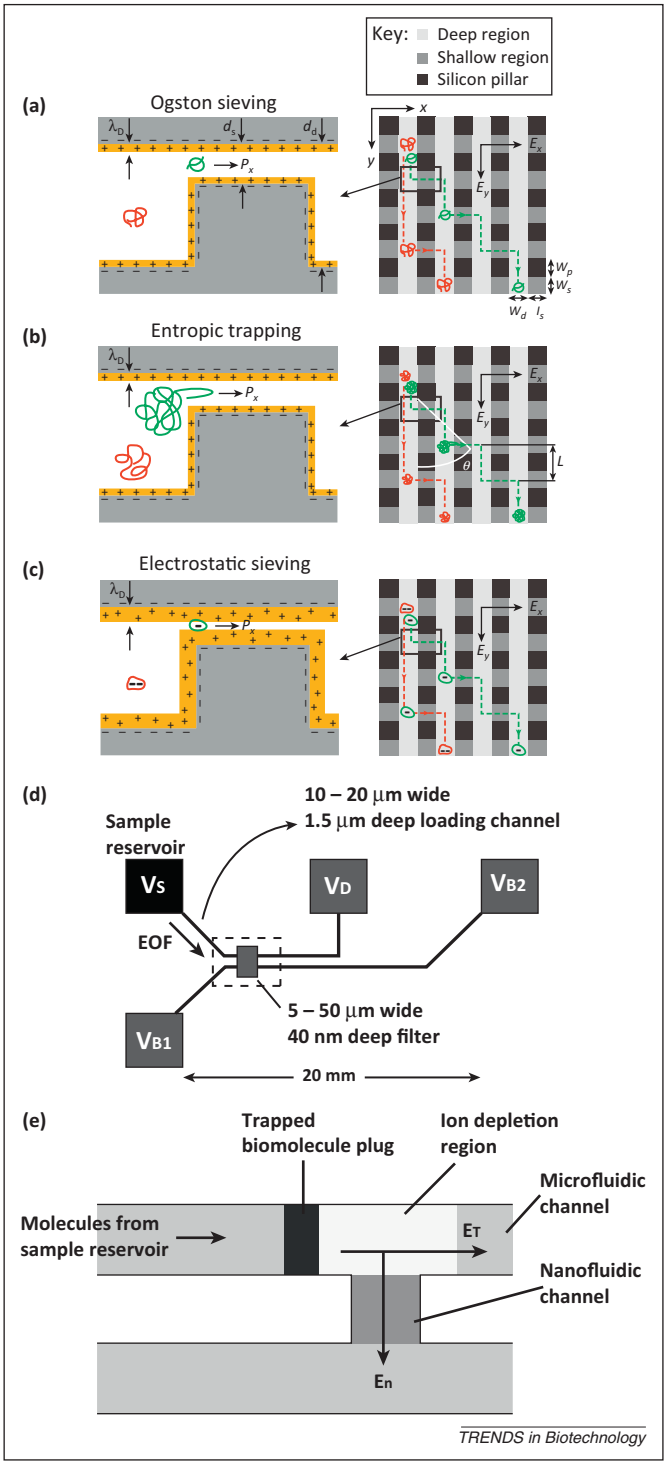

Figure 2. Schematic depiction of how nanofluidic devices aid in proteomic analysis. Left panel shows a microfabricated anisotropic sieving structure that consists of a 2D periodic nanofluidic filter array. The nanofluidic protein concentration device collects and traps molecules efficiently. The anisotropic nanofilter array (ANA) causes different sized or charged biomolecules (such as proteins, DNA, and carbohydrates) to be separated by deep region. **(a,b)** When the Debye length λ_D < ds (Debye layer highlighted in yellow), the steric exclusion effect dictates jump dynamics. For Ogston sieving (a), smaller-sized molecules (in green) are preferred for nanofilter passage, resulting in a greater nanofilter jump passage rate P_x than large-sized molecules (in red). In entropic trapping (b), the longer linear molecules (in green) assume a greater probability of hernia formation and thus a greater passage rate P_x than the short molecules (in red). **(c)** Electrostatic sieving becomes dominant when λ_D = ds. Similar sized globular molecules with a lower negative net charge (in green) experience lesser electrostatic repulsion than those with more negative charge when crossing the negatively-charged nanofilter, resulting in a greater passage rate P_x. The mean drift distance L between two consecutive nanofilter crossings plays a determinant role for the migration trajectory, with a shorter L leading to a larger stream deflection angle θ, where θ is defined with respect to the positive y axis. **(d)** The layout of the nanofluidic protein concentration device. **(e)** The schematic diagram of the device. The device efficiency is based on the electrokinetic trapping and nonlinear electroosmotic flow in the chip with nanofluidic filters (5–50 μm wide, 40 nm deep). The filters filled with buffer solution are used as an ion-selective membrane to generate an ion depletion region for electrokinetic trapping. The electrical field En is used to generate the ion depletion region and extended space charge layer that traps biomolecules. ET is used to generate the electroosmotic flow to bring the molecules into the trapped biomolecule plug from the reservoir.

identify changes in activation of extracellular signal-related kinase-1 (ERK1) and ERK2, mitogen-activated kinase-1 (MEK), signal transducer and activator of transcription protein-3 (STAT3) and STAT5, c-Jun N-terminal kinase (JNK), and caspase-3 in imatinib-treated chronic myelogeneous leukemia (CML) cells; measure an unanticipated change in the phosphorylation of an ERK2 isomer in individuals with CML who responded to imatinib; and detect a decrease in STAT3 and STAT5 phosphorylation in individuals with lymphoma who were treated with atorvastatin. Therefore, nanofluidics is a new and highly sensitive method for determining oncoprotein expression and phosphorylation in clinical specimens and for development of new therapeutic biomarkers for cancer.

Concluding remarks

Nanoproteomics has technical advantages over traditional proteomics. (i) Target proteins bind to the functionalized carbon nanotubes for real-time detection of biomolecules based on changes in electrical conductance of the device. (ii) QDs act as a labeling agent for sensitive detection of target proteins in an array format. Protein analytes are detected by monitoring the emission spectra of bound QDs. (iii) Silver and gold nanoparticles improve sensitivity as a result of the increased local electric field on the nanoparticle surface and the electronic coupling between adsorbed molecules and the surface of the gold nanoparticle. (iv) Target proteins are specifically detected when the conductance of a specific device is altered after target proteins bind to the silicon nanowires of the device; conductance of the other device remains unaltered.

The combination of nanotechnology with proteomic analysis will be of significant importance in developing miniaturized analytical nanomaterials, including separation media and channels at nanoscale levels for biomedical research [60,61]. The development of methods such as nanoelectrophoresis and nanochromatography gives hope that single molecules rather than concentration parameters could be dealt with in the near future. The unique properties of nanostructures (e.g., nanoparticles made from noble metals) could be the superior alternatives to conventional fluorescent dyes and lead to a breakthrough in proteomics. Nanofluidics offers a means to handle a very small volume of samples. The combination of nanofluidics with nanostructured labels is a powerful tool for diagnosis of diseases and their prognosis. Separation- and label-free nanosensors with multiplexing capacities could also alleviate the complexity problems that proteomic analysis is confronting in terms of shape and composition of nanomaterials. Integrating these new nanotechnology methods with the traditional proteomic methods can help to resolve proteomic complexity.

Nanotechnologies are converting microarrays into nanoarrays to help to isolate a single cell and may eventually make detection of single protein molecules possible by the use of nanomaterials such as QDs. Although still in its immature stage, significant advances have been made in nanoscale proteomic analysis because manipulating single molecules is the subject of nanotechnology. Without any doubt, the marriage of the two subjects and the new nanoproteomics will revolutionize proteomics research in the future.

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References

1 Xu, Y. *et al.* (2010) Porous polymer monolithic column with surface-bound gold nanoparticles for the capture and separation of cysteine-containing peptides. *Anal. Chem.* 82, 3352–3358
2 O'Farrell, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250, 4007–4021
3 Righetti, P.G. (2004) Bioanalysis: its past, present, and some future. *Electrophoresis* 25, 2111–2127
4 Peterson, D.S. *et al.* (2003) Dual-function microanalytical device by *in situ* photolithographic grafting of porous polymer monolith: integrating solid-phase extraction and enzymatic digestion for peptide mass mapping. *Anal. Chem.* 75, 5328–5335
5 Geiser, L. *et al.* (2008) In-line system containing porous polymer monoliths for protein digestion with immobilized pepsin, peptide preconcentration and nano-liquid chromatography separation coupled to electrospray ionization mass spectroscopy. *J. Chromatogr. A* 1188, 88–96
6 Archakov, A.I. *et al.* (2007) AFM fishing nanotechnology is the way to reverse the Avogadro number in proteomics. *Proteomics* 7, 4–9
7 Chandra, H. and Srivastava, S. (2010) Cell-free synthesis-based protein microarrays and their applications. *Proteomics* 10, 717–730
8 Wepf, A. *et al.* (2009) Quantitative interaction proteomics using mass spectrometry. *Nat. Methods* 6, 203–205
9 Liao, L. *et al.* (2008) Quantitative proteomic analysis of primary neurons reveals diverse changes in synaptic protein content in fmr1 knockout mice. *Proc. Natl. Acad. Sci. U.S.A.* 105, 15281–15286
10 Matsuoka, S. *et al.* (2007) ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* 316, 1160–1166
11 Jia, L. *et al.* (1996) S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control. *Nature* 380, 221–226
12 Simon, D.I. *et al.* (1996) Polynitrosylated proteins: characterization, bioactivity, and functional consequences. *Proc. Natl. Acad. Sci. U.S.A.* 93, 4736–4741
13 Jia, L. *et al.* (2005) Pharmacoproteomic effects of isoniazid, ethambutol, and N-geranyl-N′-(2-adamantyl)ethane-1,2-diamine (SQ109) on *Mycobacterium tuberculosis* H37Rv. *J. Pharmacol. Exp. Ther.* 315, 905–911
14 Bell, A.W. *et al.* (2009) A HUPO test sample study reveals common problems in mass spectrometry-based proteomics. *Nat. Methods* 6, 423–430
15 Stevens, T.S. and Langhorst, M.A. (1982) Agglomerated pellicular anion exchange columns for ion chromatography. *Anal. Chem.* 54, 950–953
16 Jia, L. (2005) Nanoparticle formulation increases oral bioavailability of poorly soluble drugs: approaches experimental evidences and theory. *Curr. Nanosci.* 1, 237–243
17 Liang, X.J. *et al.* (2008) Biopharmaceutics and therapeutic potential of engineered nanomaterials. *Curr. Drug Metab.* 9, 697–709
18 Benes, M.J. *et al.* (2005) Methacrylate-based chromatographic media. *J. Sep. Sci.* 28, 1855–1875
19 Nilsson, C. *et al.* (2007) Use of nanoparticles in capillary and microchip electrochromatography. *J. Chromatogr. A* 1168, 212–224
20 Cintron, J.M. and Colon, L.A. (2002) Organo-silica nano-particles used in ultrahigh-pressure liquid chromatography. *Analyst* 127, 701–704
21 Cristea, I.M. *et al.* (2005) Fluorescent proteins as proteomic probes. *Mol. Cell. Proteomics* 4, 1933–1941
22 Qu, Q.S. *et al.* (2008) Gold microspheres modified with octadecanethiol for capillary liquid chromatography. *J. Chromatogr. A* 1198–1199, 95–100
23 Zhong, Z. *et al.* (2003) More recent progress in the preparation of Au nanostructures, properties, and applications. *Anal. Lett.* 36, 3097–3118
24 Wang, A. *et al.* (2006) Gold nanoparticle-assisted protein enrichment and electroelution for biological samples containing low protein concentration–a prelude of gel electrophoresis. *J. Proteome Res.* 5, 1488–1492
25 Liu, T. *et al.* (2004) High-throughput comparative proteome analysis using a quantitative cysteinyl-peptide enrichment technology. *Anal. Chem.* 76, 5345–5353
26 Mukhopadhyay, K. *et al.* (2003) Gold nanoparticles assembled on amine-functionalized Na−Y zeolite: a biocompatible surface for enzyme immobilization. *Langmuir* 19, 3858–3863
27 Gygi, S.P. *et al.* (2002) Proteome analysis of low-abundance proteins using multidimensional chromatography and isotope-coded affinity tags. *J. Proteome Res.* 1, 47–54
28 Wang, S. and Regnier, F.E. (2001) Proteomics based on selecting and quantifying cysteine containing peptides by covalent chromatography. *J. Chromatogr. A* 924, 345–357
29 Ijima, S. (1991) Helical microtubules of graphitic carbon. *Nature* 354, 56–58
30 Wei, B.Q. *et al.* (2002) Microfabrication technology: organized assembly of carbon nanotubes. *Nature* 416, 495–496
31 Dai, H. (2002) Carbon nanotubes: opportunities and challenges. *Surf. Sci.* 500, 218–241
32 Bahr, J.L. *et al.* (2001) Functionalization of carbon nanotubes by electrochemical reduction of aryl diazonium salts: a bucky paper electrode. *J. Am. Chem. Soc.* 123, 6536–6542
33 Wildgoose, G.G. *et al.* (2006) Chemically modified carbon nanotubes for use in electroanalysis. *Microchim. Acta* 152, 187–214
34 He, P. *et al.* (2006) Applications of carbon nanotubes in electrochemical DNA biosensors. *Microchim. Acta* 152, 175–186
35 Wang, J. (2005) Carbon-nanotube based electrochemical biosensors: a review. *Electroanalysis* 17, 7–14
36 Merkoçi, A. *et al.* (2005) New materials for electrochemical sensing VI: carbon nanotubes. *TrAC Trends Anal. Chem.* 24, 826–838
37 Gooding, J.J. (2005) Nanostructuring electrodes with carbon nanotubes: A review on electrochemistry and applications for sensing. *Electrochim. Acta* 50, 3049–3060
38 Valcarcel, M. *et al.* (2007) Role of carbon nanotubes in analytical science. *Anal. Chem.* 79, 4788–4797
39 Katz, E. and Willner, I. (2004) Biomolecule-functionalized carbon nanotubes: applications in nanobioelectronics. *Chemphyschem* 5, 1084–1104
40 Chen, R.J. *et al.* (2003) Noncovalent functionalization of carbon nanotubes for highly specific electronic biosensors. *Proc. Natl. Acad. Sci. U.S.A.* 100, 4984–4989
41 Dyke, C.A. and Tour, J.M. (2004) Covalent functionalization of single-walled carbon nanotubes for materials applications. *J. Phys. Chem. A* 108, 11151–11159
42 Li, Y. *et al.* (2005) Incorporation of single-wall carbon nanotubes into an organic polymer monolithic stationary phase for mu-HPLC and capillary electrochromatography. *Anal. Chem.* 77, 1398–1406
43 Ugarov, M.V. *et al.* (2004) MALDI matrices for biomolecular analysis based on functionalized carbon nanomaterials. *Anal. Chem.* 76, 6734–6742
44 Chen, W.Y. *et al.* (2004) Carbon nanotubes as affinity probes for peptides and proteins in MALDI MS analysis. *J. Am. Soc. Mass Spectrom.* 15, 1629–1635
45 Li, X. *et al.* (2007) Enrichment of peptides from plasma for peptidome analysis using multiwalled carbon nanotubes. *J. Sep. Sci.* 30, 930–943
46 Johnson, C.J. *et al.* (2008) Proteomics, nanotechnology and molecular diagnostics. *Proteomics* 8, 715–730
47 Gaspari, M. *et al.* (2006) Nanoporous surfaces as harvesting agents for mass spectrometric analysis of peptides in human plasma. *J. Proteome Res.* 5, 1261–1266
48 Geho, D. *et al.* (2005) Pegylated, streptavidin-conjugated quantum dots are effective detection elements for reverse-phase protein microarrays. *Bioconjug. Chem.* 16, 559–566

49 Fu, J. *et al.* (2005) A nanofilter array chip for fast gel-free biomolecule separation. *Appl. Phys. Lett.* 87, 263902
50 Zeng, Y. and Harrison, D.J. (2007) Self-assembled colloidal arrays as three-dimensional nanofluidic sieves for separation of biomolecules on microchips. *Anal. Chem.* 79, 2289–2295
51 Jokerst, J.V. *et al.* (2009) Nano-bio-chips for high performance multiplexed protein detection: determinations of cancer biomarkers in serum and saliva using quantum dot bioconjugate labels. *Biosens. Bioelectron.* 24, 3622–3629
52 Natarajan, S. *et al.* (2008) Optimal conditions for protein array deposition using continuous flow. *Anal. Chem.* 80, 8561–8567
53 Natarajan, S. *et al.* (2008) Continuous-flow microfluidic printing of proteins for array-based applications including surface plasmon resonance imaging. *Anal. Biochem.* 373, 141–146
54 Fu, J. *et al.* (2007) A patterned anisotropic nanofluidic sieving structure for continuous-flow separation of DNA and proteins. *Nat. Nanotechnol.* 2, 121–128
55 Austin, R. (2007) Nanofluidics: a fork in the nano-road. *Nat. Nanotechnol.* 2, 79–80
56 Yamada, M. *et al.* (2009) Rapid quantification of disease-marker proteins using continuous-flow immunoseparation in a nanosieve fluidic device. *Anal. Chem.* 81, 7067–7074
57 Wang, Y.C. *et al.* (2005) Million-fold preconcentration of proteins and peptides by nanofluidic filter. *Anal. Chem.* 77, 4293–4299
58 Wang, Y.C. and Han, J. (2008) Pre-binding dynamic range and sensitivity enhancement for immuno-sensors using nanofluidic preconcentrator. *Lab Chip* 8, 392–394
59 Fan, A.C. *et al.* (2009) Nanofluidic proteomic assay for serial analysis of oncoprotein activation in clinical specimens. *Nat. Med.* 15, 566–571
60 Guihen, E. and Glennon, J.D. (2003) Nanoparticles in separation science–recent developments. *Anal. Lett.* 36, 3309–3336
61 Zhang, Z. *et al.* (2006) Applications of nanomaterials in liquid chromatography: opportunities for separation with high efficiency and selectivity. *J. Sep. Sci.* 29, 1872–1878
62 Old, W.M. *et al.* (2005) Comparison of label-free methods for quantifying human proteins by shotgun proteomics. *Mol. Cell. Proteomics* 4, 1487–1502
63 Ito, T. *et al.* (2001) A comprehensive two-hybrid analysis to explore the yeast protein interactome. *Proc. Natl. Acad. Sci. U.S.A.* 98, 4569–4574
64 Kocher, T. *et al.* (2009) High precision quantitative proteomics using iTRAQ on an LTQ Orbitrap: a new mass spectrometric method combining the benefits of all. *J. Proteome Res.* 8, 4743–4752
65 Yu, X. *et al.* (2006) Carbon nanotube amplification strategies for highly sensitive immunodetection of cancer biomarkers. *J. Am. Chem. Soc.* 128, 11199–11205
66 Kong, X.L. *et al.* (2005) High-affinity capture of proteins by diamond nanoparticles for mass spectrometric analysis. *Anal. Chem.* 77, 259–265
67 Li, Y. *et al.* (2007) Immobilization of trypsin on superparamagnetic nanoparticles for rapid and effective proteolysis. *J. Proteome Res.* 6, 3849–3855
68 Yao, N. *et al.* (2008) Enrichment of peptides in serum by C(8)-functionalized magnetic nanoparticles for direct matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis. *J. Chromatogr. A* 1185, 93–101
69 Kluchova, K. *et al.* (2009) Superparamagnetic maghemite nanoparticles from solid-state synthesis – their functionalization towards peroral MRI contrast agent and magnetic carrier for trypsin immobilization. *Biomaterials* 30, 2855–2863
70 Lin, S. *et al.* (2008) Novel microwave-assisted digestion by trypsin-immobilized magnetic nanoparticles for proteomic analysis. *J. Proteome Res.* 7, 1297–1307
71 Qin, W. (2007) Silica nanoparticles as pseudostationary phase for protein separation. *Electrophoresis* 28, 3017–3023
72 Luck, M. *et al.* (1998) Analysis of plasma protein adsorption on polymeric nanoparticles with different surface characteristics. *J. Biomed. Mater. Res.* 39, 478–485
73 Gessner, A. *et al.* (2002) Influence of surface charge density on protein adsorption on polymeric nanoparticles: analysis by two-dimensional electrophoresis. *Eur. J. Pharm. Biopharm.* 54, 165–170
74 Cedervall, T. *et al.* (2007) Detailed identification of plasma proteins adsorbed on copolymer nanoparticles. *Angew. Chem. Int. Ed. Engl.* 46, 5754–5756
75 Jia, W. *et al.* (2006) $CaCO_3$-poly(methyl methacrylate) nanoparticles for fast enrichment of low-abundance peptides followed by $CaCO_3$-core removal for MALDI-TOF MS analysis. *Angew. Chem. Int. Ed. Engl.* 45, 3345–3349
76 Lok, C.N. *et al.* (2006) Proteomic analysis of the mode of antibacterial action of silver nanoparticles. *J. Proteome Res.* 5, 916–924
77 Cao, Q. *et al.* (2010) Polymer monoliths with exchangeable chemistries: use of gold nanoparticles as intermediate ligands for capillary columns with varying surface functionalities. *Anal. Chem.* 82, 7416–7421
78 Lu, Z. *et al.* (2010) Mesoporous TiO(2) nanocrystal clusters for selective enrichment of phosphopeptides. *Anal. Chem.* 82, 7249–7258
79 Zhou, H. *et al.* (2007) Highly specific enrichment of phosphopeptides by zirconium dioxide nanoparticles for phosphoproteome analysis. *Electrophoresis* 28, 2201–2215
80 Lee, I.S. *et al.* (2006) Ni/NiO core/shell nanoparticles for selective binding and magnetic separation of histidine-tagged proteins. *J. Am. Chem. Soc.* 128, 10658–10659