ELSEVIER

Mesopore-assisted profiling strategies in clinical proteomics for drug/target discovery

Rocco Savino and Rosa Terracciano

Department of Health Sciences, University of Catanzaro Magna Græcia, Europa Avenue, Germaneto, 88100 Catanzaro, Italy

Mass spectrometry (MS)-profiling of human bodily fluids is a new approach for the discovery of novel disease biomarkers and, consequently, of new druggable targets. However, the complexity and the high dynamic range of biological samples make the characterization of endogenous peptides and/or proteins a challenging task. To this end, the introduction of new technologies, enabling sample pre-fractionation and/or pre-treatment before MS, could be useful. Progress in the field of nanostructured materials has provided innovative devices, particularly those based on mesoporous silica, which have proved to be successful. The ability to address new emerging material-based MS-profiling platforms will ultimately determine how deeply nanotechnology and proteomics can contribute to improve drug and/or target discovery.

Introduction

Mass spectrometry (MS) is undoubtedly the gold standard tool for proteomic applications. By using MS tools it is easily possible to generate a spectrum of peaks from a clinical sample, and the ability to screen and discover multiple biomarkers simultaneously opens up new avenues for early diagnosis and predictive medicine. Molecular barcodes or signatures 'written' in blood or in other bodily fluids could be revealed by the power of MS-based profiling in combination with bioinformatics approaches. Not dismissing, but embracing, the seminal concept and philosophy launched by Petricoin *et al*. [1], many research groups have addressed the strengths and weaknesses of this strategy. So, one promising field of clinical proteomics is now represented by surface-enhanced laser desorption ionization (SELDI-TOF) and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS-based approaches for profiling clinical samples.

This task can be challenging, in fact differential expression profiling requires a sensitive technology to discern every little difference and a high-throughput system to process the large series of samples necessary to reach statistical significance. The complexity and high dynamic range of such biological samples (i.e. plasma, serum, induced sputum, saliva) make the characterization of metabolites as well as endogenous peptides and proteins a challenging task. Moreover, the limitation regarding the dynamic range of mass spectrometers makes the analysis of complex bodily fluids such as plasma or serum difficult. Reducing the complexity of clinical samples could be a possible way to overcome this limitation. To this end, the assessment of new materials enabling sample pre-fractionation, or sample pre-treatment before MS profiling, could be of great benefit. Among the unconventional methods, the use of nanoporous materials has recently captured the attention of many research groups, because these systems can directly interact and selectively capture biologically significant subproteomes from complex clinical matrices. In this review we will discuss a new emerging MS-profiling tool: mesoporous silica (MPS)-assisted fractionation coupled to MALDI-TOF MS, highlighting its potentials, limitations and possible concerns in relation to drug and/or target discovery.

MS-profiling strategies for drug and/or target discovery

Proteomics provides a different approach from that of gene expression profiling, delivering a more direct method to biomarker discovery. Proteomics tools can assess protein abundance, post-translational modifications, localization and interactions with other biomolecules. Therefore, proteomic biomarkers directly reflect drug action on biological systems. However, owing to the large dynamic range and the transient nature of protein

Corresponding author: Terracciano, R. (terracciano@unicz.it)

1359-6446/06/$ - see front matter doi:10.1016/j.drudis.2011.10.002

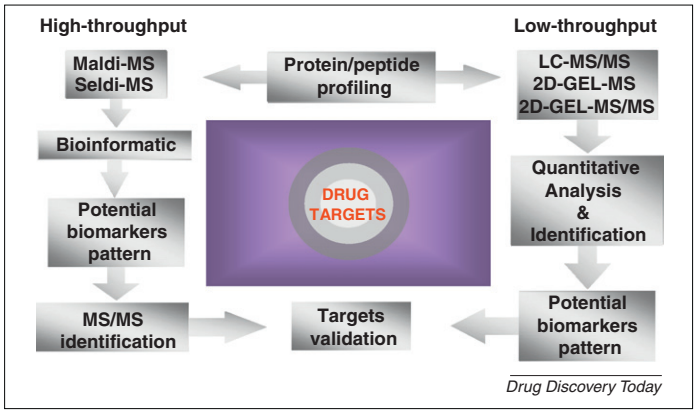

FIGURE 1

MS-based approaches could be divided in two different groups in the area of clinical drug discovery: high-throughput and low-throughput. High-throughput strategies are mainly based on MALDI-TOF MS and SELDI-TOF MS, whereas low-throughput strategies are referred to LC or 2D-GEL (2D-PAGE) coupled to MS or MS/MS.

modifications and interactions, it is difficult to identify protein biomarkers and develop stable biomarker assays [2].

Clinical proteomics comprises MS-based analytical tools that can be used to: (i) discover biomarkers of disease, (ii) identify therapeutic targets, and (iii) furnish diagnostic and prognostic information that could eventually lead to treatment decisions [3].

MS-based profiling approaches, which are at the basis of clinical proteomics, are largely dominated by two-dimensional-polyacrylamide gel electrophoresis (2D-PAGE; also known as 2D-GEL) or liquid chromatography (LC), followed by MS or tandem MS (MS/MS), and MALDI-TOF MS or its variant SELDI-TOF MS. Each of these approaches offers unique advantages but also suffers from several drawbacks. As shown in Fig. 1, the major technological platforms used in proteomics have been divided into two main categories: high and low throughput. In particular, the term 'high throughput' is commonly used to describe MALDI-TOF or SELDI-TOF MS biomarker discovery and it does not truly apply to 2D-PAGE or LC coupled with MS analysis.

Low-throughput strategies

Protein analysis by 2D-PAGE or LC, followed by MS or MS/MS analysis, is a labour-intensive and time-consuming process. 2D-PAGE followed by MS or MS/MS is not suitable for full automation and so it does not allow the rapid screening of a large number of samples, which is one of the main requirements for large-scale proteomics studies. LC coupled to MS/MS (LC–MS/MS) [4,5] is a powerful proteomics platform for the identification and the characterization of novel protein biomarkers related to human diseases. In this approach, complex protein mixtures are first digested to the peptide level using a sequence-specific protease. The resulting peptides are separated by single- or multi-dimensional LC and are then analysed by MS/MS. However, the huge number of peptides resulting from enzymatic digestion increases sample complexity, thus requiring separation in two or more chromatographic dimensions before MS/MS analysis. This process is laborious and time-consuming so, even with full automation, the LC–MS/MS-based approach can only be applicable to a limited number of samples. In fact, the identification of more than 1000 proteins in a serum or plasma sample takes days per sample [6].

High-throughput strategies

MALDI-TOF MS is a fast and versatile method for analysing small and large polypeptides contained in biological samples. Some intrinsic properties of the technique include its tolerance toward salts and buffers, high sensitivity and mass accuracy, low sample consumption, spectral simplicity owing to singly charged ions and minimal fragmentation because MALDI is a 'soft' ionization technique. Another advantage of MALDI-TOF is given by the possibility of obtaining high-resolution spectra of peaks by the use of reflectron and delay time [7]. The possibility of obtaining high-resolution spectra is an important feature, especially when a complex mixture of peptides with similar molecular weights (MWs), for example serum, plasma, urine, cerebrospinal fluid, nipple aspirate, induced sputum, among others, is being analysed for biomarker discovery. In MALDI-TOF analysis peptides are analysed more efficiently compared with larger proteins, which are difficult to ionize, therefore such peptidomic profiling studies of bodily fluids or of clinical samples are more suitable than protein profiling with MALDI. For tandem instruments, the presence of a second analyser, more commonly a second TOF (MALDI-TOF/TOF), adds sequencing capability to MALDI-MS/MS instruments, enabling more-specific peptide identification than is obtained by simple peptide-mass fingerprinting in MALDI-TOF MS [8].

As shown in Fig. 1, in a typical MS-based biomarker discovery approach, according to high-throughput strategy, the MS-based pattern recognition uses bioinformatics tools to compare MALDI (or SELDI) profiles from diseased versus healthy individuals. Given the large dataset generated by spectrum profiles, sophisticated mathematical algorithms need to be applied to recognize biomarker patterns suitable for distinguishing both classes. When the pattern displays prominent differences between the diseased and normal samples identification of individual discriminatory peaks is performed by MALDI-MS/MS analysis [9]. In this case, only relatively low MW peptides (3500–4000 Da) are easy to identify using MALDI-MS/MS sequencing. Larger peptides and proteins, however, are not yet directly identifiable and need to be partially purified and identified using microscale protein purification and in-gel digestion [10] or more-sophisticated mass spectrometers. An important drawback is the limited dynamic range of protein concentration detectable by MALDI-TOF MS (about two orders of magnitude), consequently the detection of low-abundance components in complex mixtures is a challenging task and requires pre-analytical fractionation steps. Moreover, the application of this technique to perform accurate quantitative analysis is hampered by the poor reproducibility of signal intensities, showing the need to standardize several variables that can affect the results of protein profiling [11].

An emerging application of MALDI-TOF is 'imaging MS', which enables the analysis of proteins and the determination of their distribution directly in tissue sections [12]. Basically, a frozen sample is sectioned, placed on the MALDI target plate, coated with matrix and analysed according to profiling and imaging protocols. In the profiling protocol discrete areas of tissue are analysed and the resulting peptide and/or protein profiles are subjected to biostatistical analysis to compare normal versus tumour or control versus treated tissues. In the imaging protocol an entire tissue section is analysed using an ordered array of spots,

in which spectra are acquired at intervals that define the image resolution. The intensities of all the signals are plotted as a function of their x, y co-ordinates, to obtain 2D ion intensity maps. From the resulting images it is possible to assess protein localization differences between and among samples rapidly [13]. Imaging MS has proved to be useful for imaging of small molecules, such as lipids and drug metabolites [14].

SELDI-TOF MS, is one of the most suitable approaches for profiling low MW (<20 kDa) proteins and peptides. In fact, it has been widely used in proteomics studies and, as result of its high throughput, has become a promising tool in the search for diagnostic patterns of disease [15–17]. Nevertheless, the method suffers from several drawbacks that prevent its application as a routine tool in clinical diagnostics [18]. The disadvantage of SELDI-TOF MS in peptidomics experiments is that its resolution is usually low (i.e. many peptides can exist within each ion peak so that the ion peaks of interest cannot be directly assigned) [19]. Another weak point of the technique is the poor reproducibility, within and between laboratories. In this regard, some efforts have been made to establish standardized protocols between research groups [20]. Moreover, SELDI-TOF instruments cannot directly identify candidate biomarkers because they lack MS/MS capabilities. Concerns also arise with the sensitivity of the technique and the specificity of SELDI-discovered biomarkers. The main feature of SELDI technology (i.e. the possibility of directly applying samples to the chip surface without preliminary treatments or fractionation steps) could also be seen as a drawback when compared to MALDI-TOF MS. In fact, in the MALDI-based approach, the sample preparation step is uncoupled from the MALDI MS analysis, enabling independent optimization of sample handling and the use of various MALDI MS and MS/MS instrument platforms [21,22].

Sample fractionation and/or preparation are crucial steps to generate reproducible and high quality mass spectra, which are essential prerequisites for successful proteomic pattern analysis. Therefore, development of proteome fractionation approaches that enable the acquisition of reproducible profiles and optimization and/or standardization of sample preparations find a more suitable interface with MALDI-TOF MS analysis.

Relevance of peptidome analysis and profiling

Sample fractionation and/or preparation not only help in generating reproducible and high quality mass spectra but also enable the isolation of a particular subproteome: the peptidome. Indeed, until recently, the peptidome was considered by the scientific community simply as the pool of the proteolytically derivatized peptide products that results from the action of active proteases, with an informational content equivalent to biological 'trash' or 'noise'. However, during the past five years, a novel concept has been emerging, suggesting that the peptidome could instead be a goldmine for the discovery of novel biomarkers [23,24].

In fact, four decades of research in biochemistry and molecular biology demonstrated that proteases, by exploiting exquisitely controlled cleavages at precise locations, participate in basically every biological process: the most obvious examples are apoptosis, cell-cycle progression and blood clot formation, but a fundamental part is also played by proteases in angiogenesis, morphogenesis and tissue remodelling, neuronal outgrowth, differentiation and migration, immunity, and cell proliferation [25]. Therefore, the levels of the peptides present in bodily fluids and derived from protein degradation can reflect the activity of the protease(s) generating them, which could in turn be influenced by various biological events. Thus, the levels of certain peptides can be used as indirect sensors of the biological state of an individual and, in the end, could provide invaluable information for clinical diagnoses [26]. The rationale for such a biomarker discovery strategy stems from the observation that the activity of several proteases is regulated in different disease states, and this generates a difference in the degradation pattern of the target proteins. As a consequence, the pattern of peptides resulting from the degradation process not only distinguishes between physiological and pathological states but it could also be used to uncover details in the pathological mechanisms, thus suggesting potential therapeutic options [25]. For example, Villanueva *et al*. [27] described not only cancer-specific but also cancer-type-specific peptide patterns and were able to demonstrate a direct link between peptide marker profiles of disease and differential protease activity. The patterns thus identified might have clinical use as surrogate markers for detection and classification of cancer [27]. As another example, we recently demonstrated that a peptide with m/z 2918, most probably derived from proteolysis of a yet unidentified human basic salivary proline-rich protein 2, is significantly downregulated in induced sputum obtained from chronic obstructive pulmonary disease (COPD) or asthma patients as compared with control individuals [28]. It is intriguing that a peptide derived from a protein most probably produced by the salivary glands is significantly altered in a disease (such as COPD or asthma) that develops mainly within the airways. One tempting speculation is that the pathological state alters the expression and/or activation of one or more protease(s) in the sputum; when sputum and saliva unavoidably mix during sample collection [29] the altered protease(s) determines the significant change in the levels of peptide 2918 that we detected.

The case described above is a good example of the potential usefulness of the peptidome analysis and profiling compared with protein profiling in respect to biomarker discovery. In fact, contrary to proteases or the progenitor protein, peptides are likely to show better permeability between tissue and cell membranes owing to their lower MW and their hydrophobicity patterns [30]. Therefore, the likelihood of 'fishing' proteolytic fragments in bodily fluids is expected to be significantly higher than it is for the precursor proteins.

MPS-based profiling strategies

Among well established methods for sample preparation such as classical solid phase extraction (SPE) [31] and magnetic beads (MB) [32–34], new separation and/or fractionation platforms are based on innovative application of traditional and novel materials for selective capture of low MW peptides and polypeptides from bodily fluids and tissues [28,35–53].

Among these new emerging material-based approaches we will focus mainly on MPS [28,36,40–53].

Recently, researchers from Fudan University (Shanghai, China) launched a new trend in proteomic analysis: reporting for the first time the remarkable enrichment of low-abundance peptides and proteins by the mean of zeolite nanocrystals (nanozeolites) for

direct MALDI-TOF MS analysis [35]. In the simple protocol described, the nanozeolites were incubated in a diluted solution of standard peptides or peptide digests and, after centrifugation, the peptide/nanozeolite pellets were rinsed with water and resuspended in acetonitrile and an aliquot of the slurry, mixed with MALDI matrix, was directly analysed by MALDI-TOF MS. Owing to the high adsorption capacity of the nanozeolites, after enrichment peaks of all peptides became clearly apparent even at a peptide concentration as low as 1 pg/ml. Additionally, also in the presence of high salt concentrations, MALDI-TOF MS analysis revealed stronger m/z signals with higher signal-to-noise ratios than those enriched in the absence of salt.

With this approach, the group established the proof-of-concept of the nanopore-assisted MS profiling. Owing to their small pore size (<2 nm), nanozeolite pre-concentration was demonstrated to be useful for small-sized peptide enrichment from diluted solutions. The rational consequence of this pioneering work was to test the efficiency of MPS with larger pore diameters in a range from 2 to 50 nm and with a higher surface area. The growing amount of literature in this field [28,36,40–53] is a clear indication that MPS constitute a more advantageous and suitable tool compared with nanozeolite materials for such proteomic purposes, i.e. selectively extracting endogenous peptides (the so-called peptidome or the low proteome) from a complex biological matrix and then profiling and analysing them with MS.

MPS materials constitute a new generation of materials that show ordered arrangements of channels and cavities of different geometry built up from SiO_2 unities [54]. The pore size is variable ($2\ nm < \Phi p < 50\ nm$) and can be controlled and modified, in a reasonable range, using *in situ* [55] and *ex situ* [56] synthetic strategies. Another important feature is the large surface area of the pores, which accounts for up to 95% of the total surface of the material [42]. MPS find many uses in catalysis, metal-ion extraction and optical applications. Moreover, silica particles with highly ordered mesostructures, for example MCM-41 and SBA-15, have been widely applied in the fields of separation and adsorption [57,58]. Recently, the use of trypsin immobilized onto MPS has captured the attention of many research groups, because these systems can significantly speed-up protein digestion [59].

MPS particles

In a pioneering study, our group explored a new application for MPS by developing a strategy based on MPS MCM-41 (Table 1) in combination with MALDI-TOF MS for profiling low MW plasma peptides [36]. On the basis of a molecular cut-off mechanism, we used MPS as sponges to capture peptides present in human bodily fluids. By the means of controllable pore size and surface properties (Table 1), MPS used in our first experiments were used to harvest plasma peptides, excluding from the adsorptive processes large and highly abundant proteins such as albumin and immunogobulins [36]. Continuing our investigations on MPS, we have recently developed a new MPS bead (MSB)-functionalized platform for bodily fluid fractionation [43]. Here, the large surface area of MPS is covered by a high concentration of silanol groups, which causes the MPS to resemble a 'forests of silanols'. So, starting with the consideration that the selectivity of MPS might be finely tuned by introducing chemical functional groups on the surface of MPS materials (in our case SBA-15 material; Table 1), we grafted MSBs with different functionalities such as aminopropyl, *N*-(2-aminoethyl)-3-aminopropyl, and *N*,*N*,*N*′tris(carboxymethyl)ethylenetris(carboxymethyl)ethylenediamine (Table 1). Depending on the chemical functionality introduced on the mesoporous surfaces, we tested our new platform for resolving human plasma and urine peptidome. A fine modulation of the peptidome repertoire extracted from these specific bodily fluids was obtained, thus providing rich, complementary and reproducible fingerprints, which could be useful for biomarker discovery. In Fig. 2, the workflow of the derivatized and the non-derivatized MSBs for MALDI-TOF analysis of the plasma and urine peptidome is shown. It is divided into four main steps: adsorption, separation, washing and extraction. In the first step, a small amount of particles is suspended in plasma or urine and left to act as a sponge. During this step, only peptides and proteins with sizes smaller than or comparable to the pore size are adsorbed into the mesochannels. Moreover, given the specific chemical functionality present on the mesoporous surface, the adsorption process will also be driven by electrostatic interactions. After the incubation, the separation of the beads from the supernatant occurs by gentle centrifugation, to avoid diffusion and dispersion of adsorbed peptides. Afterwards, in the washing step, salts and other contaminants, which can interfere with MALDI-TOF MS analysis, are eliminated with aqueous solution. In the final step, the peptides adsorbed onto MPS are eluted directly with the matrix solution for MALDI-TOF.

To speed up the analysis and to circumvent the risk of sample loss, we refined the previous protocol by direct spotting of the MSB-loaded suspension (slurry) on the MALDI target plate [47]. A significant enhancement in signal intensity as well as in the number of peaks was detected in the new suspension procedure. Inspection of sample morphology of the two different preparations on the MALDI plate by scanning electron microscopy (SEM) correlated the shape of the crystals obtained (in particular the surface-to-volume ratio) with the efficiency of the desorption/ionization process.

We have demonstrated that our approach in combination with MALDI-TOF technology is sensitive enough to detect plasma [36] or urine [43] peptides in the low ng/ml range. Moreover, we proved the robustness and the analytical reproducibility of our MSB approach (~10% CV for plasma, urine and induced sputum profiles within and between run replicates). Additionally, the linearity of the methodology was also demonstrated by the use of internal standards, with a dilution series of standard peptides in human plasma [36] and urine [43]. Functionalized MSBs can be readily prepared by a simple and low-cost procedure; the protocol is fast and easy to perform (Table 1) for processing bodily fluids before MS analysis, thus minimizing the possible degradation of biological samples. Overall robustness, reproducibility and linearity together with high-throughput processing suggest that the mass spectral readouts generated by this platform can be part of possible diagnostic profiles to detect significant differences between disease-specific groups. We strongly believe that this platform could be suitable for a discovery workflow of diagnostic patterns (see Fig. 3) and preliminary biomarker discovery studies are in progress. To this end, we have recently demonstrated the ability of this platform to discriminate between the 'sputome' of patients with asthma and COPD, and between the sputome of these groups and that of healthy control subjects [28]. In this above mentioned

TABLE 1

A comparison of mesoporous materials performances for proteome/peptidome profiling

MPS	Pore size (nm)	Surface area (m^2/g)	MPS amount (mg)	Biological sample	Volume of intact sample (μl)	Incub. time (h)	Ref.	MS[a]
Sil A (MCM-41)	2.6	406	5	Human plasma	100	1	[36]	MALDI-TOF
			2	Human urine	125[b]	1	[43]	
			2	Human induc. sputum	15	1	[28]	
Sil B (MCM-41)	2.7	848	5	Human plasma	100	1	[36]	MALDI-TOF
			2	Human urine	125[b]	1	[43]	
			2	Human induc. sputum	15	1	[28]	
SBA-15-APTES	4.3	273	5	Human plasma	100	1	[36]	MALDI-TOF
			2	Human urine	125[b]	1	[43]	
			2	Human induc. sputum	15	1	[26]	
SBA-15-AAPTES	4.4	310	5	Human plasma	100	1	[36]	MALDI-TOF
			2	Human urine	125[b]	1	[43]	
			2	Human induc. sputum	15	1	[28]	
SBA-15-TED	7.0	171	5	Human plasma	100	1	[36]	MALDI-TOF
			2	Human urine	120[b]	1	[43]	
			2	Human induc. sputum	15	1	[28]	
MCM-41	2.05	871	10	Human plasma	1000	2	[40]	MALDI-TOF &
				Human plasma pH 2.5	1000	2	[41]	LC–MS/MS
MCM-41(0)	2.1	771	5	Mouse liver extract	200[c]	2		MALDI-TOF &
SCX-MCM-41	4.2	460	5	Mouse liver extract	200[c]	2	[42]	LC–MS/MS
SAX-MCM-41	3.7	356	5	Mouse liver extract	200[c]	2		
Alkyl-diol@SiO_2 MCM-41[d]	2.19	781	10	Human plasma	500	7	[44]	MALDI-TOF
Alkyl-diol-vinyl MCM-41[e]	1.93	869	10	Human plasma	500	7	[46]	MALDI-TOF
MSC[f]	2.0	–	–	Human serum	10	0.5	[45]	MALDI-TOF
	5.2	–	–	Human serum	10	0.5		
	7.4	–	–	Human serum	10	0.5		
	9.0	–	–	Human serum	10	0.5		
MSC[f]	6.3	499	–	Human serum	10	0.5	[48]	MALDI-TOF
	3.9	191	–	Human serum	10	0.5		
	3.7	641	–	Human serum	10	0.5		
	3.7	1131	–	Human serum	10	0.5		
Fe_3O_4·nSiO_2 ·meso-hybrid-C8	3.7	324	0.2	Human serum	20	1	[49]	MALDI-TOF
Fe_3O_4@nSiO_2@mSiO_2	2.3	365	1.0	Rat brain extract	200[g]	1	[51]	LC–MS/MS
C8-Fe_3O_4@mSiO_2	3.5	162.5	0.2	Human serum	50	0.5	[52]	MALDI-TOF
			1.0	Mouse brain extract	400[h]	1		LC–MS/MS

[a] MS used for the analysis.
[b] 4-Fold concentrated human urine sample.
[c] Total protein concentration of 2.1 mg/ml.
[d] Alkyl-diol is present onto the exterior surface.
[e] Alkyl-diol is present onto the exterior surface, vinyl group is present on the interior surface.
[f] Mesoporous silica chip (MSC).
[g] Total protein concentration of 2.3 mg/ml.
[h] Total protein concentration of 1.1 mg/ml.

pilot study based on an MSB–MALDI-TOF/TOF MS approach, six *m*/*z* peaks have emerged as potential diagnostic peptidic patterns, able to differentiate these inflammatory airway diseases in the sputome range. Human α-defensins (HNP1, HNP2 and HPN3) and three C-terminal amidated peptides, one of which is phosphorylated on a serine residue, have been identified by MALDI-TOF/TOF MS. These findings could contribute to defining a high-throughput screening MS-based platform for monitoring key peptidic biomarkers for inflammatory and chronic respiratory diseases in induced sputum samples.

Recently, human plasma was successfully profiled by the means of MPS MCM-41, characterized by a 2.0 nm pore size and surface area of 871 m^2/g (Table 1) [40]. A selective extraction of plasma peptides in the range 1–12 kDa was obtained, as confirmed by MALDI-TOF MS analysis excluding high MW proteins. The procedure adopted was similar to our previous protocol [36], although a longer incubation time was adopted with a higher ratio of plasma volume per mg of MPS (Table 1). Later, the protocol was slightly modified by adjusting the pH of the plasma sample to 2.5 [41]. Interestingly, by 2D nano-LC–MS/MS analysis,

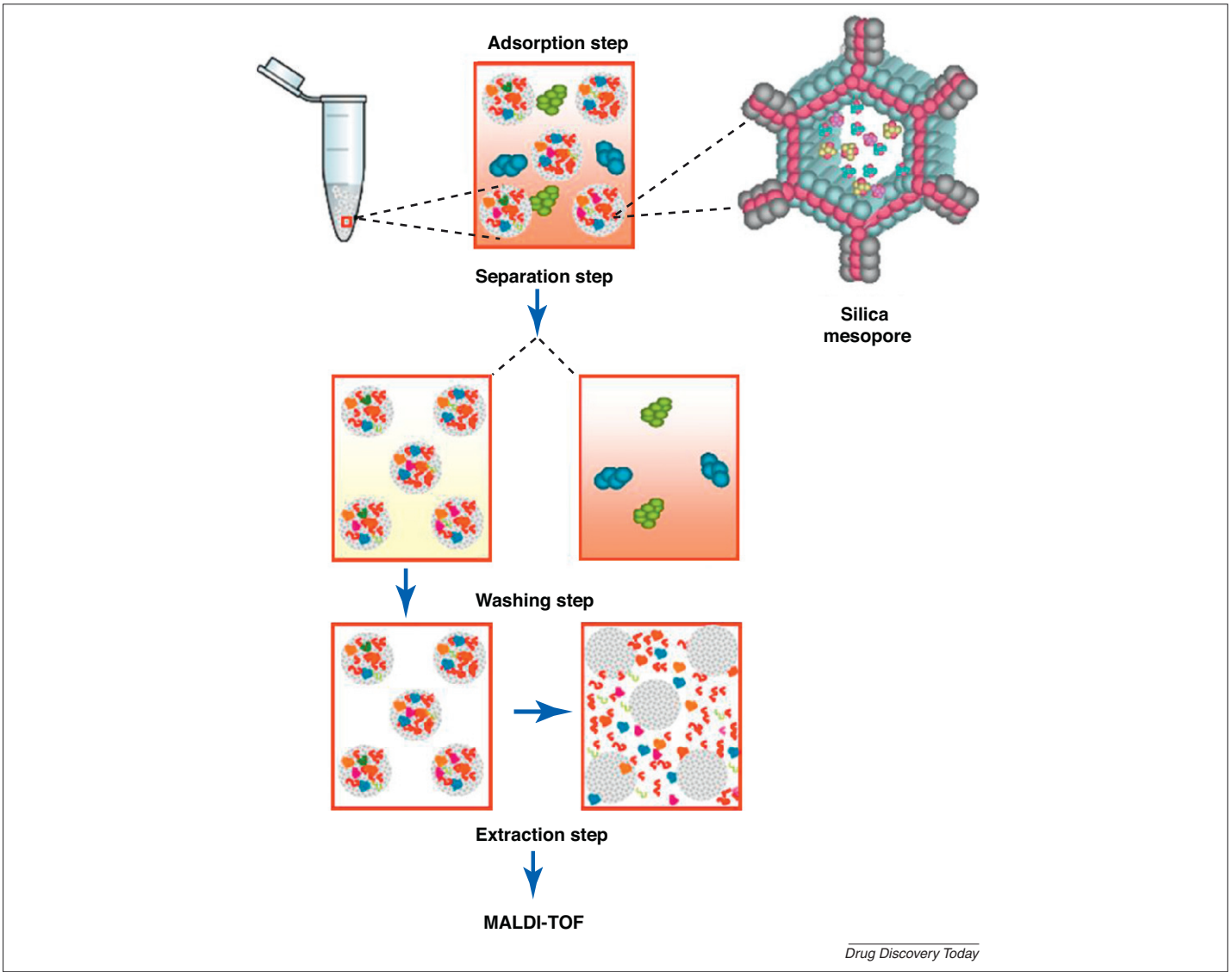

FIGURE 2

Workflow diagram of sample preparation with mesoporous silica beads (MSB). MSB are mixed with a clinical sample and shaken at room temperature for 1 h (adsorption step). The suspension is centrifuged, MSB are separated from the supernatant (separation step) and washed with aqueous solution (washing step). After the last wash, bound species are extracted and then analysed by MALDI-TOF MS.

1680 unique plasma peptides were identified in comparison with 988 identified with the previous protocol exploiting the native state of human plasma. The authors speculated that at pH 2.5 the ionization of silanols present on silica surfaces is suppressed, whereas the hydrophobic interactions between peptides and MPS inner walls increased, thus enhancing efficiency in plasma peptide enrichment. Recently, the same group introduced $–SO_3H$ and $–NH_2$ groups on MCM-41 nanoparticles, to provide strong cation- and anion-exchange features [42]. In this case, the authors tested the extraction efficiency of the new modified MCM-41 substrate on mouse liver tissue. The same amount of mouse liver extract was enriched by SCX-MCM-41 and SAX-MCM-41 and the eluted peptides were identified by 1D reverse phase (RP)–LC–MS/MS. The higher efficiency in peptide enrichment was demonstrated for SCX-MCM-41 (198 unique peptides identified) over that of SAX-MCM-41 (119 unique peptides identified). Most of the peptides enriched by SAX-MCM-41 are acidic peptides. In another experiment, the previous protocol was slightly modified replacing the unique step elution with a stepwise elution. With this new refined procedure more peptides were eluted in comparison with the previous method; in particular, more peptides were identified in the first two fractions for SCX-MCM-41 (334 and 283 unique peptides) and in the last two fractions for SAX-MCM-41 (116 and 156 unique peptides). Moreover, it is worth underlining that the data demonstrated the high capacity of the MPS substrate for peptide enrichment from complex biological samples, making this procedure suitable for large-scale sample pre-fractionation followed by nano-LC–MS/MS analysis. Finally, in an additional experiment, the same mouse liver extract was enriched in turn by SCX-MCM-41, SAX-MCM-41 and MCM-41 (pore size 2.1 nm) and the enriched fractions were analysed by 2D nano-LC–MS/MS: a total of 2721 unique peptides were identified. The data reported in this study demonstrated that MPS can be used not only to support MALDI-TOF profiling

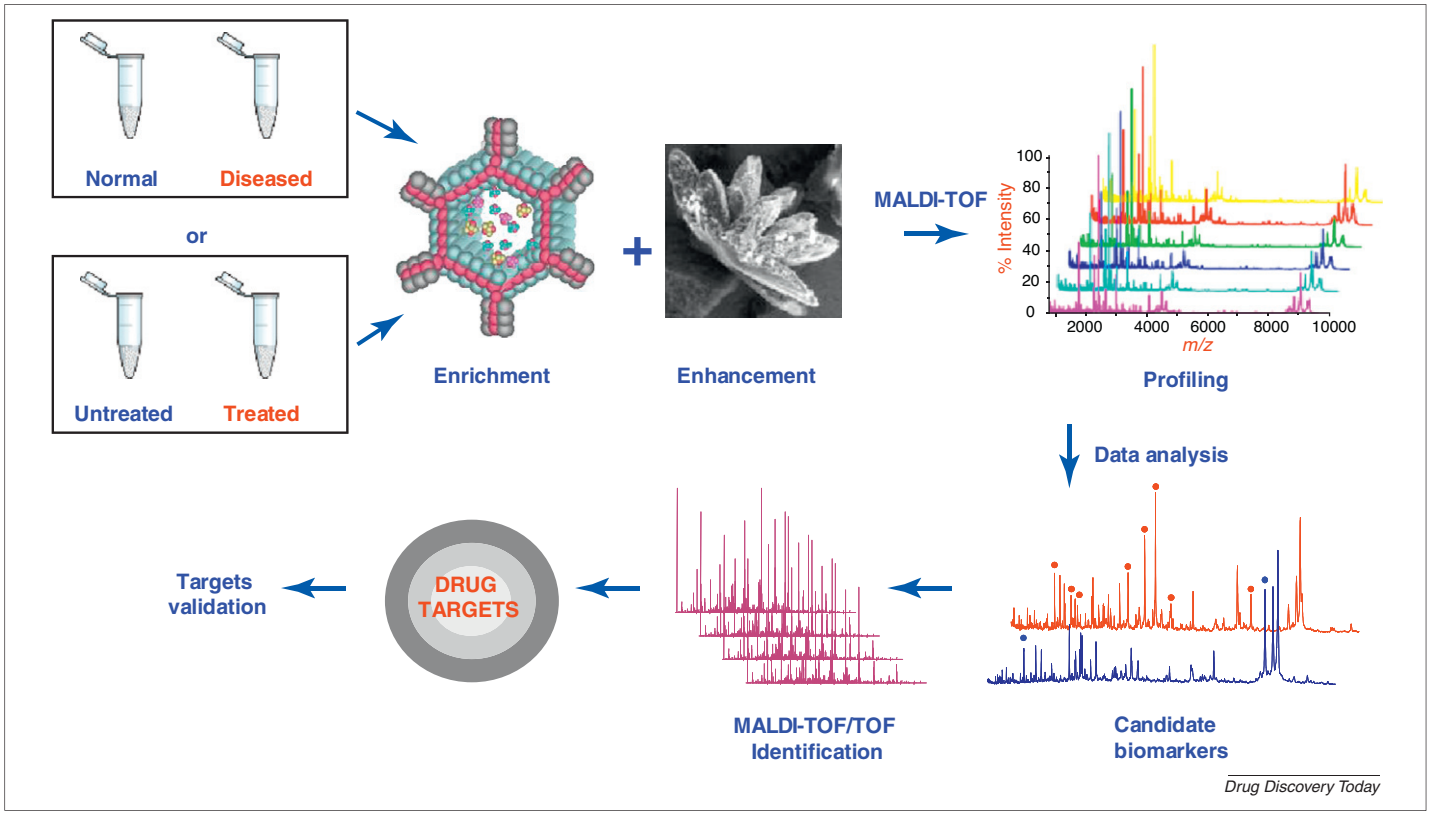

FIGURE 3

MSB–MALDI-TOF MS biomarker discovery workflow. Samples (normal versus diseased or untreated versus treated) are enriched into their low MW components through MSB. The peptidome extracted is analysed as slurry to enable signal enhancement by MALDI-TOF MS. Acquired spectra by both populations are analysed by discriminant analysis. The differentially expressed *m/z* peaks are identified by MALDI-TOF/TOF MS. The potential targets are subjected to target validation (outside of the scope of this review).

studies but are also promising for an extensive large-scale proteomic workflow based on MPS pre-fractionation of biological samples followed by LC–MS/MS analysis.

An important issue in the MPS-mediated size-selective adsorption of peptides present in a biological sample, in which larger proteins are highly abundant (plasma, serum, induced sputum, saliva, among others), is the non-specific adsorption on the external surface that might decrease the extracting selectivity and the desorbing recovery. In fact, high MW proteins could block the pore entrances. Interesting studies in this field that specifically addressed the problem of non-specific surface sorption on the outer surface of MPS were recently reported by Qi *et al.* [44,46]. First, they introduced alkyl-diol groups onto the exterior surface of MCM-41 with the aim of reducing the undesired adsorption of high MW proteins on the outer surface [44]. Later, they also modified the inner surface of MCM-41, by grafting vinyl groups which were found beneficial in reducing the irreversible protein adsorption on silanols present in the interior surface [46]. In fact, introducing these specific modifications, an improved selection of low MW (<11 kDa) over high MW proteins from human plasma was obtained in modified MCM-41 materials over the non-derivatized MCM-41. Despite the long incubation time (i.e. 7 hours; Table 1), which is a speed limitation in clinical proteomic analysis, these preliminary data might be of great interest for the optimization of such operative MPS platforms.

MPS chips

Exciting studies on the selective properties of MPS thin films toward the serum peptidome have been recently published [45,48]. Different MPS chips (MSC) were produced by the evaporation-induced self-assembly procedure, in which the porosity, the thickness and the pore alignment were precisely controlled at the nano level. A rapid 3-step on-chip protocol was at the basis of this strategy, aimed at enriching serum low MW components. In the first step (incubation), a few μl of serum (Table 1) were spotted on the chip surface; in the second step (washing), protein species excluded by the pores were removed by washing; in the final step, peptides harvested inside the pores were eluted and then analysed by MALDI-TOF MS. First, the authors demonstrated the size-dependent depletion of high MW proteins by using six kinds of MSC with pore sizes from 2.0 to 11.7 nm. In a second set of experiments, they found a differential and selective enrichment of low MW species correlated with the pore sizes. Finally, the enrichment efficiency was evaluated on human serum by comparing, from MALDI spectra, the number of *m/z* peaks of serum low MW proteins eluted from MSC. The number of peaks detected on crude serum (i.e. 109) increased after fractionation on MSC. The highest number of serum peaks (i.e. 225) was harvested by MSC with a pore size of 7.6 nm and with 57.2% porosity, whereas the lowest number (i.e. 167) was detected for MSC with 3.5 nm pore size and with 53.9% porosity. MSC with pore sizes of 2 nm and

11.7 nm were not used for serum experiments. Additionally, by merging the spectrum from each MSC eluate a total of 365 non-redundant peaks were detected [45]. In depth experiments were also performed to assess the conservation and the long-term stability of analytes harvested in the porous array of the MSC. In fact, the MSC incubated with human serum and dried after washing were stored for three weeks at room temperature. MALDI-TOF MS spectral readouts of the protein and/or peptide species eluted were similar to those of freshly fractionated serum. Through further studies [48], the same authors investigated how the structural variation of the MSC might affect the selectivity and the recovery of human serum low MW proteins. In particular, averages of 90, 89 and 75 peaks for 3D cubic, 3D honeycomb hexagonal and 2D hexagonal nanoscale morphologies were detected, respectively, for the *m*/*z* range from 800 to 10 000. The higher recovery efficiency for both 3D nanoporous morphologies was attributed by the authors to the increased pore connectivity and the reduced steric hindrance imposed on the diffusion of the peptides. Interestingly, in this study the authors derivatized the chip surface with amine, thiol, carboxyl and epoxy groups, demonstrating that chemical functionalization of MSC by cationic and anionic groups could provide a further increase of the specificity and the selectivity of peptide enrichment. To this end, a solution of 26 standard peptides and proteins with different MWs and isoelectric points was used. However, experiments regarding the selectivity and/or specificity of serum peptide enrichment by these derivatized MSC have not been reported.

MPS microspheres

In conclusion, we wish to acknowledge recently published literature on hybrid magnetic MPS microspheres, rationally conceived for the enrichment of peptides before the direct analysis by MALDI-TOF MS [49–52]. Although several kinds of magnetic beads are well established in proteomic workflow analysis [32–34], the magnetic microspheres described in these studies have the new feature of being surrounded by a MPS film, rendering them innovative in the field of peptidomics. In particular, Sun *et al.* prepared microspheres, in which a magnetic core (Fe_3O_4) was surrounded by a middle nonporous silica layer, covered by an ordered mesoporous hybrid outer layer functionalized with octyl hydrophobic groups. Such microspheres, characterized by a surface area of 324 m^2/g with a pore diameter of ~3.7 nm and a pore volume of 0.304 cm^3/g, have shown good enrichment performance, especially for tryptic digests of rat cerebellum proteins and endogenous peptides of crude human serum (see also Table 1). In this case, after the enrichment, many peaks from MALDI-TOF MS were preferentially detected in the *m*/*z* range from 1500 to 5000 [49].

Initially conceived as efficient adsorbents for removal of microcystins [50], superparamagnetic microspheres, composed of an Fe_3O_4@nSiO_2 core and of a perpendicularly aligned mesoporous SiO_2 shell (pore size: 2.3 nm; see also Table 1), have been recently applied to large-scale enrichment of endogenous peptides in rat brain extract [51]. Peptides eluted from these microspheres were analysed by 1D nano-LC–electrospray ionization (ESI)-MS/MS analysis. A total of 60 unique peptides were identified with a MW up to 4500 Da; in particular, the majority of the peptides identified with high confidence showed a MW in the range of 1200–2400 Da.

Very recently, Liu *et al.* prepared magnetic MPS microspheres with C8-modified interior pore walls (C8-Fe_3O_4@mSiO_2), with mesopores of 3.49 nm and a surface area of 162.5 m^2/g [52]. These microspheres showed a better performance in extracting mouse brain peptides when compared with those previously described, enabling the identification of 267 peptides [52]. The presence of C8 alkyl chains (responsible of hydrophobic interactions) and the bigger pore diameters (see Table 1) probably form the basis of these results.

From a brief analysis in terms of the number of peptides extracted and of the MALDI-TOF spectral readouts reported in the literature, our opinion is that the efficiency of the extraction protocols based on the magnetic MPS microspheres is slightly lower than that based on MPS particles. Because the studies described above are preliminary, many other efforts are needed to enhance the peptidic yield of these devices. It could be that the capacity of the magnetic MPS microspheres can be improved further by increasing the thickness of the mesoporous phase and/or varying the textural properties of it. By contrast, the protocols based on magnetic microspheres described in this section are quick and easy to perform: after the incubation with the biological sample solution, the microspheres are separated from the solution with the help of a magnetic field, then rinsed by water and eluted. Therefore, magnetic MPS microspheres are convenient microtools that offer the advantage of facilitating the separation steps owing to their magnetic properties. Additionally, they are more amenable to automation and miniaturization.

MPS-based profiling for phosphorylated peptides and proteins

It is well known that phosphorylation is a post-translational modification that has a key role in cellular signalling pathways, molecular recognition, cellular metabolism, among others. [60]. The detection of phosphorylated peptides and/or proteins and the identification of phosphorylation sites represent a challenging task in proteomics, owing to their low abundance in complex biological mixtures and their acidic nature [61]. Among the several approaches proposed during the past few years, immobilized metal-ion affinity chromatography (IMAC) [62–66] and metal oxide affinity chromatography (MOAC) [67–72] seem to be powerful tools for selectively concentrating phosphopeptides and/or phosphoproteins.

The use of mesoporous materials other than silica has also been recently applied to the selective enrichment of phospopeptides [70,73–76]. MPS also have been tested for phosphopeptide and/or phosphoprotein enrichment [53]. In this study, Hu and co-workers synthesized titanium(IV)-immobilized MPS particles to enrich endogenous phosphopeptides from human serum selectively. In particular, MPS MCM-41 particles were derivatized with titanium phosphonate to ensure size-exclusion adsorption and also to render them specific for phosphopeptides. Interestingly, the authors investigated the expression difference between sera of 12 hepatocellular carcinoma (HCC) patients and 12 healthy individuals, by directly enriching the phosphopeptides from sera. Comparison of MALDI-TOF profiles of enriched serum phosphopeptides showed a different expression of phosphorylated fragments degraded from fibrinogen protein between HCC patients and healthy subjects. Four discriminatory serum phosphopeptides

were then validated by the combination of isobaric tagging for relative and absolute quantitation and MALDI-TOF/TOF MS [53].

Important issues in MPS extraction strategy

A crucial issue in assessing the efficiency of MPS as selective adsorbents, particularly for trace or less abundant peptides (which could be potential pathological biomarkers), is to prove that an effective adsorption occurs inside the mesopore, with no leaching and/or non-specific surface sorption on the outer surface of MPS. It has been reported that, when the mesopore diameter is sufficiently large to accommodate biomolecules, proteins penetrate into the mesoporous networks, as well as being adsorbed onto the external surface [77].

Another important point to consider when working with MPS is the complexity of the biological sample. Therefore, different experimental parameters (pH, incubation time, desorbing solution, among others) have to be varied to obtain the optimal peptide recovery. In fact, for comparative profiling studies in clinical proteomics more m/z features might be translated for a higher probability of discovering a potential pattern of disease biomarkers.

The rational derivatization of the MPS surface has a crucial role in finely tuning the electrostatic interactions between biomolecules and MPS. Additionally, by the mean of appropriate functional groups grafted on the surface of MPS, it is possible to address the selectivity toward such post-translational modification (PTM), as demonstrated by the above mentioned study by Hu *et al.* [53]. Developing devices that enable selective isolation of low MW peptides, in particular endogenous peptides with PTMs, before MS analysis could accelerate the discovery rate of potential biomarkers and drug targets. However, further investigation is needed regarding this new emerging sample preparation strategy. Although intriguing, the great majority of the described experimental procedures based on MPS lacks in standardization and reproducibility of MS analysis, which represents crucial tasks for potential clinical applications.

Concluding remarks

In the research area of clinical proteomics, proteome and peptidome MS-profiling of human bodily fluids constitutes a new approach for the discovery of novel disease biomarkers and consequently new druggable targets.

MPS offers an excellent platform for harvesting the low MW fraction of complex biological fluids or tissue. The unique features of these materials, such as the presence of an ordered array of pores with narrow pore size, the high surface area nearly 95% of the total and the chemical and the mechanical stability, make them exceptional devices for a selective and high capacity recovery of potential biomarkers from tissue, bodily fluids and clinical specimens, limiting any possible degradation.

The possibility of controlling the pore size as well as the tailoring of surface chemistry could enable the selective adsorption of the subproteome, for example endogenous phosphopeptides. Additionally, the quick and easy protocols described in the literature reviewed here demonstrate the full compatibility between MALDI-TOF MS and LC–MS/MS analysis, ensuring highly reproducible profiles. However, because the era of mesoproteomics is just in its infancy, only a few preclinical studies have been reported to demonstrate how the MSP–MALDI-TOF approach can identify differential peptide profiles between healthy and disease groups of individuals.

The simple and fast extraction protocols, the high sensitivity, reproducibility and the capacity of the MPS are surely the keys to the success of this upcoming separation technology. Therefore, we argue that in the near future the fruitful combination of proteins and/or peptide enrichment by MPS with MALDI-TOF MS could serve as a novel useful tool for a reliable and high-throughput proteomic analysis.

References

1 Petricoin, E.F. *et al.* (2002) Use of proteomic patterns in serum to identify ovarian cancer. *Lancet* 359, 572–577
2 van Gool, A.J. *et al.* (2010) From biomarker strategies to biomarker activities and back. *Drug Discov. Today* 15, 121–126
3 Dhamoon, A.S. *et al.* (2007) The ongoing evolution of proteomics in malignancy. *Drug Discov. Today* 12, 700–708
4 Swanson, S.K. and Washburn, M.P. (2005) The continuing evolution of shotgun proteomics. *Drug Discov. Today* 10, 719–725
5 Chen, G. and Pramanik, B.N. (2009) Application of LC/MS to proteomics studies: current status and future prospects. *Drug Discov. Today* 14, 465–471
6 Ye, X. *et al.* (2009) Targeted proteomics for validation of biomarkers in clinical samples. *Brief Funct. Genomic Proteomic* 8, 126–135
7 Hortin, G.L. (2006) The MALDI-TOF mass spectrometric view of the plasma proteome and peptidome. *Clin. Chem.* 52, 1223–1237
8 Aebersold, R. and Mann, M. (2003) Mass spectrometry-based proteomics. *Nature* 422, 198–207
9 Pusch, W. *et al.* (2003) Mass spectrometry-based clinical proteomics. *Pharmacogenomics* 4, 463–473
10 Semmes, O.J. *et al.* (2005) Discrete serum protein signatures discriminate between human retrovirus-associated hematologic and neurologic disease. *Leukemia* 19, 1229–1238
11 Villanueva, J. *et al.* (2005) Correcting common errors in identifying cancer-specific serum peptide signatures. *J. Proteome Res.* 4, 1060–1072
12 Chaurand, P. *et al.* (2005) Imaging mass spectrometry: principles and potentials. *Toxicol. Pathol.* 33, 92–101
13 Reyzer, M.L. and Caprioli, R.M. (2007) MALDI-MS-based imaging of small molecules and proteins in tissues. *Curr. Opin. Chem. Biol.* 11, 29–35
14 Hsieh, Y. *et al.* (2007) Mapping pharmaceuticals in tissues using MALDI imaging mass spectrometry. *J. Pharmacol. Toxicol. Methods* 55, 193–200
15 Pitteri, S.J. and Hanash, S.M. (2007) Proteomic approaches for cancer biomarker discovery in plasma. *Expert Rev. Proteomics* 4, 589–590
16 Hodgetts, A. *et al.* (2007) Biomarker discovery in infectious diseases using SELDI. *Future Microbiol.* 2, 35–49
17 Whelan, L.C. *et al.* (2008) Applications of SELDI-MS technology in oncology. *J. Cell. Mol. Med.* 12, 1535–1547
18 Diamandis, E.P. (2004) Mass spectrometry as a diagnostic and a cancer biomarker discovery tool: opportunities and potential limitations. *Mol. Cell. Proteomics* 3, 367–378
19 Zhang, X. *et al.* (2007) Mass spectrometry-based 'omics' technologies in cancer diagnostics. *Mass Spectrom. Rev.* 26, 403–431
20 Semmes, O.J. *et al.* (2005) Evaluation of serum protein profiling by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry for the detection of prostate cancer. I. Assessment of platform reproducibility. *Clin. Chem.* 51, 102–112
21 Callesen, A.K. *et al.* (2008) Reproducibility of serum protein profiling by systematic assessment using solid-phase extraction and matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* 22, 291–300
22 Navare, A. *et al.* (2008) Serum biomarker profiling by solid-phase extraction with particle-embedded micro tips and matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* 22, 997–1008

23 Liotta, L.A. and Petricoin, E.F. (2006) Serum peptidome for cancer detection: spinning biologic trash into diagnostic gold. *J. Clin. Invest.* 116, 26–30
24 Novak, K. (2006) Biomarkers: taking out the trash. *Nat. Rev. Cancer* 6, 92
25 Hu, L. *et al.* (2009) Recent advances in mass spectrometry-based peptidome analysis. *Expert Rev. Proteomics* 6, 433–447
26 Petricoin, E.F. *et al.* (2006) The blood peptidome: a higher dimension of information content for cancer biomarker discovery. *Nat. Rev. Cancer* 6, 961–967
27 Villanueva, J. *et al.* (2006) Differential exoprotease activities confer tumor-specific serum peptidome patterns. *J. Clin. Invest.* 116, 271–284
28 Terracciano, R. *et al.* (2011) Peptidome profiling of induced sputum by mesoporous silica beads and MALDI-TOF MS for non-invasive biomarker discovery of chronic inflammatory lung diseases. *Proteomics* 11, 3402–3414
29 Nicholas, B. *et al.* (2006) Shotgun proteomic analysis of human-induced sputum. *Proteomics* 6, 4390–4401
30 Tammen, H. *et al.* (2007) Oncopeptidomics – a commentary on opportunities and limitations. *Cancer Lett.* 249, 80–86
31 Callesen, A.K. *et al.* (2009) Serum protein profiling by solid phase extraction and mass spectrometry: a future diagnostics tool? *Proteomics* 9, 1428–1441
32 Villanueva, J. *et al.* (2004) Serum peptide profiling by magnetic particle-assisted, automated sample processing and MALDI-TOF mass spectrometry. *Anal. Chem.* 76, 1560–1570
33 Zhang, X. *et al.* (2004) Evaluation of a novel, integrated approach using functionalized magnetic beads, bench-top MALDI-TOF-MS with prestructured sample supports, and pattern recognition software for profiling potential biomarkers in human plasma. *J. Biomol. Tech.* 15, 167–175
34 de Noo, M.E. *et al.* (2005) Reliability of human serum protein profiles generated with C8 magnetic beads assisted MALDI-TOF mass spectrometry. *Anal. Chem.* 77, 7232–7241
35 Zhang, Y. *et al.* (2005) Enrichment of low-abundance peptides and proteins on zeolite nanocrystals for direct MALDI-TOF MS analysis. *Angew. Chem. Int. Ed.* 44, 615–617
36 Terracciano, R. *et al.* (2006) Selective binding and enrichment for low-molecular weight biomarker molecules in human plasma after exposure to nanoporous silica particles. *Proteomics* 6, 3243–3250
37 Righetti, P.G. *et al.* (2006) Protein equalizer technology: the quest for a 'democratic proteome'. *Proteomics* 6, 3980–3992
38 Rainer, M. *et al.* (2007) Mass spectrometric identification of serum peptides employing derivatized poly(glycidyl methacrylate/divinyl benzene) particles and mu-HPLC. *J. Proteome Res.* 6, 382–386
39 Luque-Garcia, J.L. and Neubert, T.A. (2007) Sample preparation for serum/plasma profiling and biomarker identification by mass spectrometry. *J. Chromatogr. A* 1153, 259–276
40 Tian, R. *et al.* (2007) Selective extraction of peptides from human plasma by highly ordered mesoporous silica particles for peptidome analysis. *Angew. Chem. Int. Ed.* 46, 962–965
41 Tian, R. *et al.* (2007) Selective extraction of peptides in acidic human plasma by porous silica nanoparticles for peptidome analysis with 2-D LC–MS/MS. *J. Sep. Sci.* 30, 2204–2209
42 Tian, R. *et al.* (2007) Selective enrichment of endogenous peptides by chemically modified porous nanoparticles for peptidome analysis. *J. Chromatogr. A* 1216, 1270–1278
43 Terracciano, R. *et al.* (2009) Derivatized mesoporous silica beads for MALDI-TOF MS profiling of human plasma and urine. *Bioconjug. Chem.* 20, 913–923
44 Qi, Y. *et al.* (2009) Improved selection of LMW over HMW proteins from human plasma by mesoporous silica particles with external modification. *Talanta* 80, 703–709
45 Bouamrani, A. *et al.* (2010) Mesoporous silica chips for selective enrichment and stabilization of low molecular weight proteome. *Proteomics* 10, 496–505
46 Qi, Y. *et al.* (2010) Selective extraction of low molecular weight proteins by mesoporous silica particles with modified internal and external surfaces. *Anal. Bioanal. Chem.* 398, 1715–1722
47 Terracciano, R. *et al.* (2010) Enhancing plasma peptide MALDI-TOF-MS profiling by mesoporous silica assisted crystallization. *Talanta* 80, 1532–1538
48 Hu, Y. *et al.* (2010) Tailoring of the nanotexture of mesoporous silica films and their functionalized derivatives for selectively harvesting low molecular weight protein. *ACS Nano* 4, 439–451
49 Sun, L. *et al.* (2011) Octyl-functionalized hybrid magnetic mesoporous microspheres for enrichment of low-concentration peptides prior to direct analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* 25, 1257–1265
50 Deng, Y. *et al.* (2008) Superparamagnetic high-magnetization microspheres with an Fe_3O_4@SiO_2 core and perpendicularly aligned mesoporous SiO_2 shell for removal of microcystins. *J. Am. Chem. Soc.* 130, 28–29
51 Chen, H. *et al.* (2010) Selective separation and enrichment of peptides for MS analysis using the microspheres composed of Fe_3O_4@nSiO_2 core and perpendicularly aligned mesoporous SiO_2 shell. *Proteomics* 10, 930–939
52 Liu, S. *et al.* **Preparation of magnetic core-mesoporous shell microspheres with C8-modified interior pore-walls and their application in selective enrichment and analysis of mouse brain peptidome**. *Proteomics* (in press)
53 Hu, L. *et al.* (2009) Profiling of endogenous serum phosphorylated peptides by titanium (IV) immobilized mesoporous silica particles enrichment and MALDI-TOF MS detection. *Anal. Chem.* 81, 94–104
54 Beck, J.S. *et al.* (1992) A new family of mesoporous molecular-sieves prepared with liquid-crystal templates. *J. Am. Chem. Soc.* 114, 10834–10843
55 Zhao, X.S. *et al.* (1999) A novel method for tailoring the pore-opening size of MCM-41 materials. *Chem. Commun.* 15, 1391–1392
56 Firouzi, A. *et al.* (1997) Alkaline lyotropic silicate-surfactant liquid crystals. *J. Am. Chem. Soc.* 119, 3596–3610
57 Sun, J. *et al.* (2006) Ultrafast enzyme immobilization over large-pore nanoscale mesoporous silica particles. *Chem. Commun.* 28, 1322–1324
58 Hudson, S. *et al.* (2008) Proteins in mesoporous silicates. *Angew. Chem. Int. Ed.* 47, 8582–8594
59 Savino, R. *et al.* (2011) In mesopore protein digestion: a new forthcoming strategy in proteomics. *Molecules* 16, 5938–5962
60 Rust, H.L. and Thompson, P.R. (2011) Kinase consensus sequences: a breeding ground for crosstalk. *ACS Chem. Biol.* 6, 881–892
61 Smith, J.C. and Figeys, D. (2008) Recent developments in mass spectrometry based quantitative phosphoproteomics. *Biochem. Cell Biol.* 86, 137–148
62 Kokubu, M. *et al.* (2005) Specificity of immobilized metal affinity-based IMAC/C18 tip enrichment of phosphopeptides for protein phosphorylation analysis. *Anal. Chem.* 77, 5144–5154
63 Feuerstein, I. *et al.* (2005) Phosphoproteomic analysis using immobilized metal ion affinity chromatography on the basis of cellulose powder. *Proteomics* 5, 46–54
64 Smith, S.D. *et al.* (2004) Using immobilized metal affinity chromatography, two-dimensional electrophoresis and mass spectrometry to identify hepatocellular proteins with copper-binding ability. *J. Proteome Res.* 3, 834–840
65 Stensballe, A. *et al.* (2001) Characterization of phosphoproteins from electrophoretic gels by nanoscale Fe(III) affinity chromatography with off-line mass spectrometry analysis. *Proteomics* 1, 207–222
66 Zhang, X. *et al.* (2007) Highly efficient phosphopeptide enrichment by calcium phosphate precipitation combined with subsequent IMAC enrichment. *Mol. Cell. Proteomics* 6, 2032–2042
67 Larsen, M.R. *et al.* (2005) Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Mol. Cell. Proteomics* 4, 873–886
68 Thingholm, T.E. *et al.* (2008) TiO (2)-based phosphoproteomic analysis of the plasma membrane and the effects of phosphatase inhibitor treatment. *J. Proteome Res.* 7, 3304–3313
69 Zhou, H. *et al.* (2007) Highly specific enrichment of phosphopeptides by zirconium dioxide nanoparticles for phosphoproteome analysis. *Electrophoresis* 28, 2201–2215
70 Han, L. *et al.* (2008) Mesoporous Fe_2O_3 microspheres: rapid and effective enrichment of phosphopeptides for MALDI-TOF MS analysis. *J. Colloid Interface Sci.* 318, 315–321
71 Chen, C.T. and Chen, Y.C. (2005) Fe_3O_4/TiO_2 core/shell nanoparticles as affinity probes for the analysis of phosphopeptides using TiO_2 surface-assisted laser desorption/ionization mass spectrometry. *Anal. Chem.* 77, 5912–5919
72 Kweon, H.K. and Hakansson, K. (2006) Selective zirconium dioxide-based enrichment of phosphorylated peptides for mass spectrometric analysis. *Anal. Chem.* 78, 1743–1749
73 Nelson, C.A. *et al.* (2009) Mesoporous zirconium oxide nanomaterials effectively enrich phosphopeptides for mass spectrometry-based phosphoproteomics. *Chem. Commun.* 43, 6607–6609
74 Lu, Z. *et al.* (2010) Self-assembled TiO_2 nanocrystal clusters for selective enrichment of intact phosphorylated proteins. *Angew. Chem. Int. Ed.* 49, 1862–1866
75 Niklew, M. *et al.* (2010) Phosphopeptide screening using nanocrystalline titanium dioxide films as affinity matrix-assisted laser desorption ionization targets in mass spectrometry. *Anal. Chem.* 82, 1047–1053
76 Eriksson, A. *et al.* (2010) Optimized protocol for on-target phosphopeptide enrichment prior to matrix-assisted laser desorption–ionization mass spectrometry using mesoporous titanium dioxide. *Anal. Chem.* 82, 4577–4583
77 Qiao, L. *et al.* (2008) Nanoporous reactor for efficient proteolysis. *Chem. Eur. J.* 14, 151–157