Use of monolithic supports for high-throughput protein and peptide separation in proteomics

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List of abbreviations: Ab, antibody; DDA, data dependent acquisition; DIA, data independent acquisition; HIC, hydrophobic interaction chromatography; HTP, high-throughput; IMAC, immobilized metal affinity chromatography; IMER, immobilized enzyme reactor; LIT, linear ion trap; PTM, posttranslational modification; Q, quadrupole; SAX, strong anion exchange; SCX, strong cation exchange; TMT, tandem mass tag;

Keywords: high-throughput, liquid chromatography, mass spectrometry, monoliths, proteomics

Total number of words including figure and table legends: 8,954

Abstract

The exclusive properties of monolithic supports enable fast mass transfer, high porosity, low back pressure, easy preparation process and miniaturisation and the availability of different chemistries make them particularly suitable materials for high-throughput (HTP) protein and peptide separation. In this review recent advances in monolith-based chromatographic supports for HTP screening of protein and peptide samples are presented and their application in HTP sample preparation (separation, enrichment, depletion, proteolytic digestion) for HTP proteomics is discussed. Development and applications of different monolithic capillary

Received: 06 22, 2017; Revised: 09 02, 2017; Accepted: 09 03, 2017

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/elps.201700260.

columns in HTP MS-based bottom-up and top-down proteomics are overviewed. By discussing the chromatographic conditions and the mass spectrometric data acquisition conditions an attempt is made to present currently demonstrated capacities of monolithic capillary columns for HTP identification and quantification of proteins and peptides from complex biological samples by MS-based proteomics. Also, some recent advances in basic monolith technology of importance for proteomics are also discussed.

1.0 Introduction

The proteome is comprised of all expressed proteins in a sample (cells, tissues, a whole organism or biological system) their proteoforms, modification states and organization in macromolecular assembles, in a given time and space [1]. Because of the high complexity of a proteome different tools and approaches are used in proteomics methodology in order to access its high complexity. Specific identification and highly sensitive quantification of proteins, their PTMs, and protein complexes can be achieved by using antibodies and/or mass spectrometry. In that respect, we differentiate antibody-based and mass spectrometry-based proteomics [2]. Both strategies require separation of complex protein mixtures prior to detection in order to obtain comprehensive and reliable data about qualitative and quantitative composition of the sample. In the antibody-based proteomics approach the role of separation is to reduce the possibility of cross-reactivity that may introduce false positive or false negative results. In the mass spectrometry-based proteomics, the role of separation is to supply the sample (complex mixture of proteins or peptides) in the form, amount and time frame that will enable successful examination using a particular MS technique. In order to access optimal information, separation can be performed in more than one analytical dimension [3]. Concerning MS-based proteomics, different analytical dimensions can be technically combined in both offline and online mode [3]. In the offline mode, fractions from the first separation dimension are collected, treated, and individually submitted to the next dimension. The online mode includes direct coupling between different analytical dimensions and a mass spectrometer.

High-throughput protein purification or high-throughput protein screening are specially designed approaches for purification (enrichment, depletion) and/or analysis of proteins from a large number of analytical samples. On the other hand, high-throughput MS-based proteomics considers simultaneous qualitative and quantitative analysis of hundreds-thousands of proteome components (in one sample), with a high degree of reproducibility [2].

Most important techniques for separation of proteins are polyacrylamide gel electrophoresis, capillary electrophoresis and liquid chromatography, while separations of peptides are achieved mostly through CE and LC.

Widely accepted advanced polyacrylamide gel electrophoretic techniques (1D, IEF, 2D) are capable of reproducibly resolving complex protein mixtures into many hundreds of spots. Identification of proteins from these spots using antibodies can provide thousands of hits, but accuracy of identification and quantification can be compromised by the fact that one spot can contain several different protein molecules that can potentially cross-react with antibodies. Moreover, proteoforms of one particular protein can be found in different spots on the gel (or western blot membrane). Limited availability of monoclonal antibodies, due to high costs of Ab development technology, inability of development of Ab against a whole range of proteoforms basically determine Ab-based proteomics as hypothesis driven proteome analysis [4]. Identification of proteins from gel spots using MS can provide identification of thousands of highly abundant proteins. In comparison to Ab-based proteomics, MS-based proteomics enables two basically different approaches: hypothesis-driven (targeted) approach [5], and discovery based (shotgun bottom-up, as well as top-down) approach [6]. However, efficacy of PAGE techniques in proteome separation are limited due to resolution, long time of analysis, technical complexity (losses during peptide/protein extraction from the gel, low throughput, complex handling of large 2D gels) and inherent

(fundamental) issues concerning separation of small and large proteins, highly charged proteins, and low abundant proteins [3, 7]. Moreover, separation of peptides is not feasible by PAGE. The complexity of proteome and limited ability of gel electrophoretic techniques forced researchers to develop alternative approaches.

Capillary electrophoretic techniques are in focus and are constantly evolving [8] since they provide high resolution, sensitivity and reproducibility in separation of proteins and peptides from complex biological mixtures [9-11]. Interfering compounds that may be present in a protein sample (such as lipids, precipitates, etc.), are big problems in PAGE and LC, but are acceptable to a certain extent in CE [12]. The analysis time including reconditioning of CE capillary is faster than in PAGE. Two CE modes, namely capillary zone electrophoresis and capillary isoelectric focusing can be efficiently coupled to MS. When coupled to MS detection, online or offline [9, 13, 14], CE is an attractive technique for multidimensional proteome analysis both at the level of intact proteins [14] and peptides. Special advantage that certain CE techniques offer over LC separation in combination with MS detection, comes from constant composition of the liquid phase [12]. The main problems with CE application are limited amount of sample that may be loaded to a capillary and relative complexity of the technique. Also, in direct online 2D combination with LC-MS additional problem could stem from high speed of CE and sharp peaks [3, 9] and in the case of CIEF the presence of ampholytes.

Several LC techniques are used in proteomics: fast protein liquid chromatography (FPLC), HPLC, UHPLC and nano-HPLC. Materials that are used as matrices (supports, stationary phases) for protein separations in LC can be divided in two different groups, bulk materials (granules/beads/particles) and monolithic supports. Matrices are modified to enable different separation LC modes (anion or cation exchangers of different strength, reversed phase matrices of different hydrophobicity, hydrophilic matrices and affinity matrices). This is a

widely used technique for protein and peptide separations in proteomics due to good resolution and reproducibility, availability of different separation modes, ease of standardisation and automation and in the case of monolith supports, short analysis time that allows high-throughput analysis. Online coupling of one or multiple dimensions of LC to MS is the most widely accepted peptide separation technique in high-throughput MS-based proteomics [1, 3].

This review addresses some of the basic concepts of monolith synthesis and modification important for proteomic applications, advances and new developments in application of monoliths in HTP protein purification and screening, as well as its application in HTP MS-based proteomics.

2.0 General aspects of monolithic materials

Chromatography has prominent place in analytical chemistry and science of separation in general. From its birth on start of the 20th century it was a matter of trial and error and often misunderstood as it took some time for scientists to accept it due to initial failures in obtaining reproducible results. This lack of success was mostly caused by insufficient knowledge about the importance of proper preparation of stationary phase. From the moment it gained wider acceptance in scientific circles continuous research began for development of new techniques and materials that could be implemented for chromatographic uses. Monolithic column chromatography presents one of such discoveries that was first demonstrated around 1960s but it gained wider recognition around 1990s [15, 16]. Driving impetus for development of monolithic column chromatography stemmed from a niche in biomolecule separations where slow mass transfer, based on diffusion, in bulk materials was one of the main obstacles for improvement of separation efficacy. High porosity of monoliths enables substantially better mass transfer properties since in monoliths convection is the main

driving force for mass transfer while role of diffusion is minimized. Consequences of high porosity of monoliths are also low back pressure and high flow rates. These are advantageous both for analytical and preparatory applications. Concerning preparatory scale purification of biomolecules monoliths offer advantage in scaling up, and already columns up to 40 L volume are commercially available [17]. Also, they are suitable for scaling down and construction of miniature (lab-on-a-chip) analytical devices. Monoliths do not require use of frits hence additionally enable reduction of sample volume that can be handled.

Many different types of monolithic materials have been prepared [15], but only acrylate based, styrene based and silica-based ones are commercially available [18]. There is a good rationale for this as making new custom type of matrix require a hard process of determination of optimal conditions for generation of a suitable support starting from completely new set of monomers. Slight modifications of an existing monolithic matrix material or incorporation of new wanted functionalities within manufacture thereof most commonly, but not exclusively, done through surface modification of prepared monolithic matrix solid phase therefore can lead to a faster support development [19]. Some of the literatures that describe or summarize synthesis of monolithic supports are presented in table 1. Silica based columns have a foothold in research of HPLC particulate stationary phase. This has been exploited to a great extent relying on previously known chemistry of surface modification with reported success in attachment of various small and big molecules. Development of organic polymer based monoliths was initially harder task concerning preparation of material with required structural properties [19, 20]. Fortunately, inherent diversity of organic compounds allowed the utilization of a plethora of co-monomers to generate materials of equivalent if not better traits [21]. Having in mind that the main driving force for development of these techniques laid in biomedical field where electrophoretic gels based on polymerization of acrylamide are an established technique, one can only regret that

its tendency toward gel formation complicated its use for monolithic column preparation. This problem has been in detail addressed in the past and it is still an attractive topic [20, 22]. Recently, a hydrophilic polyacrylamide-based monolith was successfully applied in glycoproteomic analysis [23]. It is obvious form such starting points that RP based separation phases had large share and wide spread use in the field at least in the start. There are reports of comparison of monolithic and HPLC supports, especially since HPLC is done on commercially available bulk materials. In a recent publication authors devoted their attention to preparation of a novel monolithic support by hyper crosslinking poly(styrene-co-vinylbenzyl chloride-co-divinylbenzene) [24]. They created a material that could be used in both reversed and normal phase separations. Although their approach lead to creation of a system that is less effective in terms of plate numbers, it opens a venue for further expansion of the range of applications for monolithic columns with functionalities required for other separation mechanisms.

In addition to the previously mentioned RP-type phase, monolithic supports can take use of a variety of other interactions like: IEC [25, 26], HILIC [23, 27, 28], HIC [29], fluorinated tags separation [30], IMAC [31, 32], affinity chromatography [33], imprinted phases [34], chiral separation [35]. Although functional groups on a support determine the type of separation, an interplay within different principles can lead to a novel approach. This was aptly demonstrated in a few publications that used commercially available monolithic ion-exchange based columns and saturated them with certain metallic ions to obtain separation in IMAC mode chromatography of peptides [36, 37].

In terms of materials besides purely organic supports, there are approaches aimed at creating hybrid materials. It was reported experimenting with monolithic columns based on sulfonate ion-exchanging groups for separation of phosphopeptides as a SCX chromatography support [38]. Synthesized sulfonate-SCX hybrid monolithic column exhibited superior characteristics

to a particulate SCX column in terms of water permeability and sample loading capacity [38]. A similar problem was tackled by combining phosphonate based monolithic columns with Ti^{4+} ions an approach that mirrors previously mentioned IMAC attempts [32]. It may still be open to discussion if the matrix described in this paper falls within true organic-inorganic hybrid matrix as no clear boundary has been set in the field. The monolith presented in this work is silica based one with surface modified in such way to incorporate small chelating molecule for the Ti^{4+} ions. Comparison with Fe^{3+}-IMAC column shows expected superior performance of Ti^{4+}-IMAC due to is higher charge. Valuable study investigated the effect that the incorporation of perfluorinated compounds in monolithic matrix exerts on resolution of compounds previously tagged with similar perfluorinated tags [30]. This attempt to combine preparation of tagged enzymatic protein digests and intrinsic property of perfluorinated compounds that makes them immiscible both with water and organic liquids but readily miscible with one another allowed the monolithic matrix to interact with perfluorinated tags via perfluorinated sections in the matrix.

In order to improve shortcomings of both organic and silica based monoliths different strategies were employed. Solutions of sometime similar challenges led to approaches that merged best of silica and organic monoliths resulting in a new hybrid monolith with improved properties [39-41]. A method that combines silica based cage-like polyhedral oligomeric silsesquioxane incorporated within organic matrix was explored [39]. It remains to be seen whether or not this approach will really accomplish the envisioned goal of easy fabrication, wide pH range tolerance, good mechanical stability and high permeability or give something substantially new compared to other methods described previously. Exciting use of another inorganic material comes from the incorporation of Au nanoparticles on columns containing HS-groups in an attempt to bind them inside the monolithic matrix [42, 43]. These works describe in detail all the aspects of fabrication of such supports including the

preparation of Au particles from Au salts before application to column or their *in situ* reduction on column, as well as the level of crosslinking of the monolith itself. The possible use of the same material for both HILIC-based enrichment and IMER in direct coupling was clearly demonstrated in this work. Desorption from HILIC column is achieved by simple change of mobile phase.

Strength of monolith-based separations lies also in a great flexibility of chromatographic formats (large, small or capillary columns, guard or trap columns, SPE, spin columns, discs, tips, 96-well plates, microfluidic systems, etc.). For instance a lab on a chip system was developed for SPE, IMER (with stopped flow) and subsequent separation using an organic monolith SAX column coated with Poly-E323, a polycationic compound to reduce protein and peptide adsorption onto the capillary wall [26]. The polycationic coating and the polymer monolith materials proved to be compatible with each other, providing a high quality solid phase extraction bed and a robust coating to reduce protein adsorption and additionally generate anodal flow which is advantageous for electrospray. Reversed-phase type adsorption effects cannot be avoided in either silica based or organic based monoliths and are generally measured and controlled by choice of co-monomers. The inclusion of zwitterionic acrylate co-monomer that enables pH triggered switching between superhydrophobic and superhydrophilic properties [44] is important as it gives a material with controlled wettability and already mentioned switching regulated by the pH of the mobile phase. Material is superhydrophobic at pH 1 and 14 while it shows superhydrophilic properties at pH 4. This smart material could at least allow creating a system that could first be used as SPE at one pH of the mobile phase and as monolith chromatography system at another pH range, if not even as a fully-fledged 2D chromatography system.

Notable is also the use of “thiol-ene” for creation of switchable phases [45]. Thiol-ene strategy seems to outgrow from previously described monoliths created to be used with Au

nanoparticles. The presence of HS-group can be effectively used as scavenger for methacrylic compounds. Therefore under radical polymerization condition (induced either thermally or by UV irradiation) HS-group presents a natural growth point. Truly remarkable work exploited imprinting targeted protein surfaces on the monolith matrix for the selective capture [34]. The authors were able to show enrichment for the targeted protein compared to other proteins, and they achieved desorption of the protein from that matrix thermally without changing of solvent. Monolith matrix commonly used express and exploits to some extent RP character which can sometimes be incompatible in protein separation if preservation of the native structure and biological function of the protein is a goal. The matrix used in this paper is superior in this respect as it uses rather hydrophilic chains to encircle the protein in its native state during imprinting procedure which allow the preservation of native protein structure. Thermal desorption also can lead to protein denaturation through entropy change as transition from hydrated matrix-protein complex to hydrated free matrix and hydrated free protein can induce internal change in protein conformation. The presented monolithic matrix minimizes this effect due to the fact that at temperatures used for desorption matrix chains were not hydrated. As a result, the overall entropy change is reduced, and the influence of this factor is minimized. This work could provide technology for entrapment, enrichment and preconcentration of molecules and can be used for imprinting at the level sufficient for making a pre-column for some dedicated process use.

Most monolithic phases are created by polymerization or polycondensation (and sometimes both) of widely available monomeric compounds [40]. One of the notable exceptions we noted is a paper describing ring opening metathesis polymerization to obtain monoliths for separation of nanoparticules that use somewhat expensive compounds and chemistry [46]. It is undisputable that this technique has the ability of higher customization but also easier creation of columns more suitable for techniques like 2D-LC. Monolithic materials have been

used in combination with particle-based columns to achieve similar results [47]. In this instance monolith frits have been used as precolumns for entrapment and preconcentration on particle-based columns. Authors demonstrated that this technique can be employed to create 2D-LC separation. The ability of monoliths allows easy creation and tailoring of solid phase, as well as easy column packing (as in a classical gravitational chromatography), combined with the reusability and high separation power (as those for HPLC) provides, from a chromatographer perspective, almost a golden ration of traits [46]. However, similar number of analysis can still be achieved by using less demanding equipment and pressures then with HPLC.

Polymer-based monoliths were early used for immobilization of enzymes and fast conversion of different substrates [48]. It is noteworthy to mention the making of a micro-enzyme assay based on a monolithic column [49]. This assay uses a monolithic chelating column, Ni^{2+} ions, recombinant green fluorescent protein with thrombin cleavage site and His-tag for Ni^{2+} binding to obtain a system for detection of thrombin in rather simple and automatic way. This is yet another fine example of implementation of a known biochemical techniques in an innovative manner as it describes detection range for protease concentration within three orders of magnitude.

3.0 Monoliths for high-throughput protein purification or screening

The analysis of a large number of proteinaceous samples in a reasonably short time enables completely new advanced level in healthcare (personalized medicine), biotechnology (fast analysis and control of manufacturing processes that enables processes of high complexity to be performed) and biosciences in general (advanced methods of investigation). Exclusive properties of monoliths (discussed in previous section) make these materials particularly suitable for HTP purification and screening of proteins [50-52]. Monoliths are produced in

different forms such as microtiter plates (96-well plates), pipette tips, small columns, capillary columns, as well as microfluidic devices. These are suitable for both manual and robotic manipulation. High porosity of monoliths that enable mass transport by convection is particularly suitable for separation of large proteins >50 kDa that have low diffusion constants. The porosity and macropore size of monoliths can be tuned and optimized according to particular needs [19]. Larger pores enable high flow rates and analysis of "dirty" samples. However, when flow-through pores are larger, surface area is smaller, hence capacity is reduced [19].

With exception of size-exclusion chromatography all other LC methods (IEC, HILIC, HIC, RP, IMAC, affinity, etc.) can be performed on monoliths [52]. The most often applied type of chromatography on monoliths is elution chromatography. Also, other two types, frontal and displacement chromatography can be applied. Monoliths are particularly suitable for sample displacement chromatography of proteins. In comparison to particle-based materials, here displacement by sample components occurs at much lower loading and practically independent of flow rate and column size [53]. Two or more different monoliths can be easily combined to prepare a system suitable for conjoint chromatography. This enables exploration of different chromatographic methods and capacities in a conjoint mode [53].

Modern mass spectrometers still cannot handle a proteome dynamic range higher than 4-5 orders of magnitude. A wide dynamic range is one of the most challenging problems in MS based proteomics. Enrichment of low abundance peptide/protein or group of peptides/proteins is necessary in order to obtain amounts that will enable identification/quantification or their further applications. MS-based proteomic bottom-up analysis of phosphoproteome requires enrichment of phosphopeptides since they represent minor fraction of peptides generated by trypsinization of proteome. The Ti^{4+}–IMAC hybrid silica monolithic material was developed as attempt to improve matrix properties and

selectivity (in comparison to Fe^{3+} or Ga^{3+} -IMAC) for large-scale enrichment [54]. A procedure for fast preparation of similar monolith was recently described [55]. Another organic-based Ti^{4+}–IMAC monolith prepared in spin tip was applied for enrichment of phosphopeptides from 5 μg of trypsinized HeLa cell lysate. This enriched fraction analysed on nanoHPLC-nanoESI-Q-Orbitrap Exactive MS system with top12 DDA enabled identification of 1185 phosphopeptides [56]. The speed necessary for enrichment of phosphoproteins/phosphopeptides on hydroxyapatite was increased when monolithic column with embedded hydroxyapatite nanoparticles was used [57]. In a similar fashion TiO_2 nanoparticles were immobilized onto organic-based monolith and successfully applied for phosphopeptide enrichment [58, 59].

Different physicochemical natures of proteins and glycan components of glycoproteins require special technical and methodological approaches for glycoproteome analysis by MS. Their enrichment is still mandatory. One of the strategies for enrichment of glycoproteins/glycopeptides is affinity chromatography on stationary phases that contain immobilized carbohydrate binding proteins (lectins). The immobilization of proteins additionally reduces porosity of matrices, hence highly porous matrices, as monoliths, are preferable in order to achieve high flow rates. Molecular weight of lectins is usually up to 30 kDa, but many of them are dimers/trimers/tetramers and contain several carbohydrate binding sites. Upon binding of glycoproteins high molecular weight structures are formed, that additionally reduce flow rate and consequently speed of analysis. Taking into account this fact, the benefits with monoliths are more pronounced in enrichment of glycoproteins because they are usually high molecular weight proteins. Individual lectins or multiple lectins, were bound on different organic-based monoliths since they offer a variety of chemistries suitable for immobilization [60-62]. The limited sample capacity was a consequence of the relatively low surface area of organic-based monoliths [63]. One of the

strategies to increase the surface area is the incorporation of nanoparticles. In this way monolith can be tuned for a specific application in proteomics depending on the nature of the nanoparticle [64, 65]. The incorporation of gold nanoparticles with immobilized *Erythrina cristagalli* lectin [63] and concanavalin A [66] was employed to improve binding capacity. This monolith casted into pipette tip [63] offered a possibility for design of HTP strategies.

Enrichment of glycoproteins/glycopeptides and glycans can be efficiently performed by use of boronate-affinity chromatography. Design and preparation of boronate-affinity monolithic matrices and strategies for their application were reviewed by Li et al. [67].

One more widely applied strategy for enrichment of glycopetides is HILIC. Organic-based monoliths can provide level of hydrophilicity suitable for HILIC [23]. An amide functionalized HILIC monolith was examined for enrichment of glycopeptides from 6 μg of trypsinized HeLa cell lysate and 1 μl of human serum [23]. Enriched samples were analysed on nanoHPLC(C18)-nanoESI-LTQ-Orbitrap Velos MS system operated in top10 DDA identifying 530 (282 proteins) and 262 (124 proteins) N-glycosyalted peptides respectively. Silica-based monoliths are not hydrophilic enough for HILIC. However, hybrid organic-silica HILIC monolith was developed and examined for enrichment of glycopeptides [68]. Using the same Velos MS system, operated in top20 DDA mode, 486 (279 proteins) N-glycosylation sites were identified from three parallel analyses of samples enriched from 1 μg of proteins obtained from ~10^4 HeLa cells. An interesting HILIC monolith functionalized with glycocluster grafted β-cyclodextrin was recently produced [69]. The advantage of this monolith over several monolith and bulk materials, in respect to enrichment of glycopeptides, was demonstrated in comparative analysis on a MALDI-TOF-MS system.

Enrichment of glycoproteins/glycopeptides can be also achieved by hydrazide monoliths. However, when these monoliths are employed information about structure of glyco-component is lost.

High affinity binding of HS- groups for gold can be exploited for selective capture of cysteine-containing peptides. A monolithic column with surface-bound gold nanoparticles was successfully used in bottom-up MS-based proteomics for enrichment/depletion of cysteine-containing peptides [42].

A method for protein enrichment based on avidin-biotin interaction is widely used in proteomics. An organic-based monolithic column with immobilized avidin was prepared in fused silica capillaries exhibited enrichment efficiency that outperformed commercial avidin beads [70]. A pronounced problem with unspecific binding, when complex samples are analysed, is the requirement for further optimization of the monolith's surface. However, this problem can be tackled at the level of elution. An interesting strategy based on selective elution was recently examined [71].

Big potential of monolithic matrices for immobilization of affinity ligand has been exploited with rising number of publications in the field. The most often used methods for highly-selective enrichment of low-abundance proteins, depletion of high-abundance proteins and purification/pre-fractionation of proteins in general relays on specificity of monoclonal Ab. Different chemistries are available for their immobilization onto monoliths. Advanced procedures are developed for oriented immobilization over glycan component to improve immobilization efficacy and increase binding capacity [72].

An interesting system for ELISA detection of low-abundance proteins in specific sample such as old artistic paints was recently described. This system uses monolithic material with immobilized Ab to enrich and extract protein from this complex sample that contains number of compounds that would preclude reliable detection with ELISA [73]. Another study described rapid purification of erythropoietin from biological samples on 6 µl disposable monoliths containing immobilized anti-erythropoietin Ab [74]. Purification of transferrin [75] from human plasma was performed in HTP fashion with 96-well plate format of monolithic

support with anti-transferrin monoclonal antibody immobilized over its glycan component. A 200 µl monolith per well was employed and 300 µg of transferrin was obtained, that was enough for further HTP profiling of its N-glycans. Similar study describing HTP purification of fibrinogen from human plasma was recently published [76].

The enrichment of low-abundance proteins from a complex samples such as blood plasma/serum can be achieved by sample displacement chromatography on monolithic supports without use of expensive antibodies. Application of sample displacement chromatography in HIC mode [77] and IEC mode [78] for enrichment/depletion of proteins from human plasma were described.

Besides antibodies, monoliths can be functionalized with protein G, A or L for their selective capturing or purification. Huge cohort of plasma samples from 2298 individuals was analysed in order to determine glycosylation pattern of total IgG fraction [79]. A monolithic 96-well plate (individual bed volume 150 µl) with immobilized protein G was used for HTP purification. The entire chromatographic procedure for 96 samples, including the binding, washing and elution steps, was performed in less than 30 min. The average amount of IgG isolated from 50 µl of plasma was 640 µg, indicating that the majority of IgG in the sample was successfully captured and released. Isolation with monoliths minimized risk of loss of sialic acids due to acid hydrolysis that can occur at very low pH elution conditions this chromatography requires. Elution from monoliths occurs within seconds and therefore the pH can quickly be restored to neutrality preserving the integrity and activity of the IgG molecules. Use of a vacuum suction system for liquid transfer enables easy and efficient handling of 96-well plates [79].

In addition to the above mentioned problem with high dynamic range in MS-based proteomics, large amount of peptides that originate from individual high-abundance proteins in complex samples negatively affects ionisation of low-abundance peptides. Depletion of

high-abundance proteins is one possible strategy that enables, or at least improves, analysis of certain low abundant proteins. Recent study demonstrated removal up to 94% of HSA from cell culture media, utilized for clinical embryo growth, with monolithic column containing immobilized anti-HSA Ab [80]. Upon depletion of HSA, the number of identified cellular proteins in the analysed medium by MS increased 50%.

In a blood plasma/serum high-abundant proteins are HSA and IgG. Application of monolithic columns for removal of HSA (affinity columns with immobilized anti-HSA Ab or pseudo-affinity columns with immobilized Cibacron Blue dye) and IgG (affinity columns with immobilized protein A or G) from blood plasma/serum was described [52]. Also, combination of monoliths with two different modes, affinity with ion-exchange, in a conjoint chromatography for removal of HSA and IgG was studied [81]. However, limited specificity and nonspecific binding are problems that are still not satisfactory resolved [52].

Different IEC monoliths have been used for fractionation of serum and membrane proteins [82] and also membrane proteins from liver and hepatocellular carcinoma prior to further processing (2D-PAGE and MS analysis) [83]. There are many other successful separation examples with application of IEC such as separation of manganese peroxidase and lignin peroxidase izoenzymes [84, 85], and clothing factor IX using monolithic DEAE and QA short columns [50, 51]. IEC chromatography in combination with RP is often used for 2D separation of peptides prior to bottom-up MS analysis. This can be done on-line on automated 2D-LC system (vide infra) or of-line using monolithic tips or 96-well plates.

High porosity of monoliths enables isolation and profiling of protein aggregates, vesicles, cells and viruses [52]. Automated fast HTP extraction of exosome from multiple clinical samples with anti-CD9 antibody-coupled highly porous monolithic silica microtips was described [86] . Extracted exosomes were successfully analysed by bottom-up MS-based proteomic and 1,369 proteins were quantified.

Some of the commercially available monolithic materials and available formats are listed in table 1.

4.0 Monoliths in high-throughput mass spectrometry-based proteomics

4.1.1 Monoliths in bottom-up HTP MS-based proteomics

HTP MS-based proteomics uses two basic approaches, bottom-up approach that analyses peptides generated from proteome by means of highly specific proteases, and top-down approach that analyses intact proteins [87]). Currently, bottom-up is the dominant approach in HTP analysis of proteome structure and function [1]. The majority of bottom-up studies use trypsin that generates, mostly peptides of less than 3 kDa in size. When using electrospray ionization peptides of this size tend to generate ions with m/z less than 1500. This was well suited for older generations of mass spectrometers concerning their resolution, fragmentation technology, cycling time, ion transmission technology. Also, LC material type and pore sizes were optimized for small peptides <3 kDa [88]. The exclusive use of trypsin, as well as the applied peptide separation and MS analysis strategies could be reasons why our view of proteome still remains incomplete [88, 89]. Bottom-up proteomics improved its capabilities in line with mass spectrometry technological advances, development of new proteases and separation strategies, improvements in data collection and data analysis. In order to increase the yield of protein structure information bottom-up approach was classified according to the peptide size to bottom-up (<3 kDa), extended bottom-up (3-7 kDa) and middle-down (7-15 kDa) [88]. In a time frame of 4 hours and under carefully optimized conditions, in a single dimension LC MS/MS run of shotgun proteomic experiment, advanced commercial instrumentations could identify more than 37,000 peptides belonging to around 5,000 proteins [13]. This is about half of the expressed proteome of an average human cell line. The key-features of advanced commercial instrumentation responsible for this success are ultra-high resolution and mass accuracy of mass analyser (such as ultra-high-field Orbitrap),

improved ionisation efficiency and ion transmission optics, as well as data processing strategies. Multidimensional protein identification technology can provide more than 10,000 proteins, but operational costs, sample preparation and consumption and working time of LC-MS/MS of more than one day are still substantially high [13]. Nevertheless, depending of the question posted it may not be necessary to have such vast and deep view of proteome for a given experiment [90].

Within a 3h time span of standard shotgun LC-MS/MS analysis of a single cell line lysate more than 100,000 isotope features are eluted, likely representing peptides, and they can be detected with a high resolution MS scan. However, just 16% of these are targeted by MS/MS scan and only 9% of them are identified by "top 10" DDA [90]. Importantly, this study confirmed that efficiency of peptide separation by LC is significantly higher than capacity of DDA LC-MS/MS (concerning sequencing speed and sensitivity) to obtain MS/MS spectra of all eluted peptides [90]. Moreover, when using low resolution ion selection for MS/MS and when treating samples of high complexity, all MS/MS spectra obtained remain mixture-spectra due to co-isolation of all ions (originating from co-eluted peptides) [91].

Alternative to DDA in discovery based proteomics is DIA. This acquisition technique performs fragmentation of all sampled ions of peptides that elute from LC. In this way DDA provides a comprehensive fragment ion map of the entire range of sampled precursor-ions. This acquisition technique eliminates, to a certain extent limited by its dynamic range (currently 4-5 orders), the missing values (diagnostic ions) of DDA. Once acquired with the DIA technique data can be later refined and re-mined using either discovery based or hypothesis driven approaches. However, analysis of data obtained by DIA has strict demands in term of chromatographic reproducibility. Advantage of DIA methods is the increased visibility of low abundant and isobaric peptides, as well as peptides containing different

PTMs and as a consequence an increased identification rate of proteins containing these peptides [92].

The most commonly used LC separation mode in bottom-up proteomics directly coupled to MS is reversed phase, due to compatibility of mobile phases with ESI-MS analysis. Hence, in design of 2D LC methods or multidimensional LC, RP-LC is usually used as a last dimension before MS detection. Different particle based and monolithic RP-LC columns are commercially available [93]. However, the majority of peptide and/or protein separations in HTP-MS based proteomic research was performed on particle based materials [1].

Advantages the monoliths possess in terms of efficiency, loadability and resolution in peptide separations on-line to MS detection was realized at the beginning of this century using short (60mm) 200-100 μm ID organic polymer (PS-DVB) reversed phase columns [94]. Instantly, efficiency was further increased by reduction of column inner diameter. Tryptic digest of 0.5 μg of *Saccharomyces cerevisiae* proteins separated by means of 6.6 h long gradient on silica-based C18 monolithic column 0.6 m long with 75 μm ID and analysed by HPLC-nanoESI-LC-MS/MS system with LIT mass analyser enabled identification of 5500 peptides (>1300 proteins) in 2006 [95]. At the same time silica-based monolith column 0.25 m long with 10 μm ID provided more than 5,100 peptides (>1,300 proteins) identified in 3 h gradient from a trypsinized cell lysate of *Shewanella oneidensis* on the same type of MS system [96]. Reduction of column ID requires low flow rates, therefore the amount of liquid phase and sample were reduced. This affected the extent of ion formation resulting in increased ionisation efficacy (especially in the case of non-surface active compounds such as glycans, glycopeptides, glycoproteins and in the case of analyte solutions containing a certain amount of salt) obtaining as a consequence higher signal intensities [97]. However, reduced ID of column requires loading of a sample in a small volume creating the need for a sample to be

concentrated. The sample concentration can be achieved in off-line mode (vide infra) or on-line by use of trapping columns [98].

A phosphate monolithic SCX column was explored as trapping column coupled to particle-based RPC for analysis of yeast proteome [99]. An amount of 19 µg of the tryptic digest was trapped, eluted in 17 consecutive fractions. Each fraction was subsequently resolved on RP-LC and analysed by MS/MS operated in top 6 DDA mode. More than 5,600 unique peptides (>1,500 proteins) were identified using FDR of 0.46%. Total analysis time was longer than 32 h. Monoliths as trapping columns in the front of particle-based RPC columns could bind high amounts of sample without significant influence on pressure.

Multidimensional HPLC using particle-packed SCX column in first dimension and silica-C18 monolith in second dimension with trapping column was successfully applied in analysis of endogenous peptides form plasma and urine [100]. Lower clogging rate was noted with monolithic column and importance of detailed system performance test was stressed in order to avoid memory effect of multidimensional HPLC analysis.

Multidimensional LC system (RPC-SCX-RPC) for HTP protein quantification with on-line dimethyl labelling was developed and coupled to nanoESI-LTQ-Orbitrap MS [101]. This system consisted of capillary biphasic trap column RPC-SCX and analytical RPC column. Separation in SCX mode was performed on a methacrylate-based monolith. This system provided quantification of about 1,000 proteins in 30 h from trypsinized liver cell lysate. Similar procedure applied for analysis of leukemia cell lines on the same MS system operated in top 6 DDA mode quantified more than 1200 proteins [102]

Silica-based monoliths enable construction of much longer capillary columns than organic polymer-based due to their mechanical strength and large pores that cause high porosity (up to 5 times higher than of a particle based) and low backpressure [103, 104]. These monoliths

provide high resolution separations using long and shallow gradient. One such 41 h gradient on 3.5 m long 100 μm ID silica-C18 column was demonstrated with *E.coli* cell lysate on nanoHPLC-ESI-LTQ-Orbitrap XL MS system. Using this experimental setup, without pre-fractionation (so called "one-shot" approach), the authors identified more than 22,000 peptides (2,602 proteins) and demonstrated 5-fold larger peak response than with a silica-C18 particle-based column which requires sample pre-fractionation [105]. In the same year 2010, another study showed that the benefit of these long columns when compared to shorter ones can be achieved only with sufficiently long gradient time [106]. The authors posted a question regarding what is responsible for high number of identified peptides, increased peak capacity of the column or the time available for mass spectrometer. Such a long gradient time makes optimization very tedious. A method for the optimization of the separation conditions with a long gradient time was proposed based on peak capacity theory [107]. This method was tested on a long monolith column and particle-packed columns of different particle sizes. The results showed that certain gradient durations are necessary to observe the advantage of long monolith columns over the particle-packed ones in the regard of the number of identified peptides.

In the same year, PS-DVB capillary monolithic columns of different lengths (0.25, 0.5 and 1 m) and 200 μm ID were examined in separation of proteolytic digest of *E.coli* lysate. Even with 10 h gradient total number of identified peptides was around 2,000 [108]. However, identification was performed with low mass resolution HPLC-ESI-IT MS system and it can be assumed, according to presented peak capacity values, that much more peptides could be identified using high resolution MS systems and nanoHPLC for peptide separation. Benefits regarding the number of identified and quantified proteins due to gradient optimization and application of a two-stage gradient was subsequently demonstrated on particle-packed

capillary columns [13]. Also, column length and elution gradient length were confirmed as important parameters, under constant flow rate.

One-dimensional LC with shallow gradient without peptide pre-fractionation was proposed as alternative to on-line 2D-HPLC which combines ion exchange in the first dimension with RPC in the second dimension. This is because one-dimensional LC is faster and requires less operator working hours [107].

In 2012, one-dimensional LC-MS/MS one-shot approach (without multidimensional pre-fractionation) on 4 m long silica-based C18 monolithic 100 µm ID capillary columns allowed the identification of more than 41,000 peptides (almost 6,000 proteins) from 4 µl of HeLa cell lysate in 8 h elution gradient on nanoHPLC-Q-TOF MS system operated in "top 10" DDA [109]. In the same study efficiency of particle-packed column and monolithic column was compared in LC-MS/MS analysis of trypsinized cell lysates of HeLa and *E.coli*. Number of proteins identified using monolithic column was around 3 times higher. However, extension of dynamic range was not observed. The authors attributed the success in separation efficiency to a long column, since it was assumed that ion-suppression caused by co-eluted peptides was the main reason for inability of MS systems to achieve high quality MS and MS/MS spectra [109].

The same MS system operated under the same parameters, including the same gradient conditions for peptide elution, demonstrated great performance on a silica-C18 monolithic column, this time 2 m long, loaded with 4 µl of trypsinized human induced pluripotent stem cell lysate. This one-shot approach identified almost 99,000 tryptic peptides (around 9,500 proteins) within 3 repetitions. The authors proposed this column particularly for samples available in limited amounts, such as FACS-, laser capture microdissection- or biopsy-derived samples [110].

The same research group developed 2 m long silica monolith modified with urea functional groups to perform HILIC. One µg of tryptic peptides from HeLa was loaded in one-shot to the column and eluted in 8 h gradient to nanoHPLC-Q-TOF MS system operating in "top 10" DDA [27]. The number of identified peptides was around 12,000 (2,600 proteins). Separation of peptides on HILIC and RPC silica based monolithic column was compared. The pI values of the peptides identified in the HILIC mode were correlated with the retention times, and acidic peptides tended to be more strongly retained than basic peptides. This correlation was much less present in RPC. Intensity of MS signal was about 5 times higher in HILIC mode. This was explained by the higher content of organic phase. The number of identified proteins was about the same, but tested sample loading was maximal for HILIC. Since RPC could be loaded with 4 times more peptide material it could be assumed that much more peptides would be identified with RPC. About 40 % of identified peptides were different between HILIC and RPC [27]. However, we would stress that repeatability and reproducibility of DDA are about in that range [111].

A combination of three peptide separation methodologies (OFFGEL electrophoresis, 2D-LC and the long monolithic silica-C18 capillary column LC) was employed to increase the number of identified proteins from epidermal cells of Arabidopsis root [112]. Peptide identification was performed by ESI-LTQ-Orbitrap XL MS in top 3 DDA mode analysing three independent biological replicates for each of the three tested separation methods. As expected, the number of total identified proteins was much higher than with any of three individual methodologies. Taking into account reproducibility of DDA shot-gun approach [111] it would be also interesting to see the result obtained by each of three individual techniques repeated 9 times.

Quantitative temporal changes of *Candida albicans* proteome were explored by 6-plex TMT labelling. Separation on silica-based monolithic column (4.7 m long, 100 μm ID) using 10 h elution gradient and LTQ-Orbitrap Velos MS system operated in top 10 DDA mode enabled quantitative comparison of around 1,000 proteins [113].

Protein PTMs are of special concern in proteomics due to their importance in biological processes. They are one of several sources of the proteome complexity. More than 300 PTMs are known (www.abrf.org/delta-mass, www.uniprot.org/docs/ptmlist). MS-based proteomics is well suited for their HTP study of PTMs [1]. Analysis and specially identification of new PTMs is a challenging task that requires specific enrichment process distinct for each PTM.

In order to increase the number of identified phosphopeptides form minute amounts of samples, LC system with sulfonate monolith SCX for automated on-line sample injection was proposed [38]. Phosphopeptides from 100 μg trypsinized HeLa lysate enriched by IMAC microspheres were injected onto a LC system containing a monolithic SCX in the front of particle-based RPC column coupled to nanoESI-Q-LIT MS system operating in top 3 DDA mode with MS3 triggered by characteristic neutral losses [38]. The authors showed around 19% higher number of identified phosphopeptides using sulfonate SCX than with phosphate SCX monolithic column. This result is a consequence of higher negative charge content of sulfonate SCX column. The higher negative charge content is beneficial for phosphopeptide trapping since they have less positive charge as a consequence of attached phosphate group.

Technological advances already enabled easier analysis of methylation and phosphorylation without enrichment [1]. The phosphate monolithic SCX column coupled on-line to a particle-based RPC [114] was applied for the analysis of methylproteome of *Saccharomyces cerevisiae* [115]. Interesting strategy was employed for isotope labelling of methylation

events and around 70 of those were identified using this 2D-LC-ESI-LTQ-Orbitrap XL MS system.

Working temperature is an important factor in LC. Temperature rise influences viscosity of the mobile phase increasing the flow rate. Also, modifying the surface tension enables elution of the analyte with lower concentration of organic phase. Moreover, reduction of tailing can be observed due to the changes in interaction kinetics between analyte and matrix [93]. However, in order to successfully apply increased temperature in LC, design of preheaters for mobile phase as well as column heating system should be flowless to enable reproducibility and avoid peak distortion. Monolithic columns provide possibility for separation under high temperatures [116].

The main obstacle for silica-based columns is their application under high pH due to matrix instability, limiting the applicability for sensitive ESI-MS analysis in negative ion mode. Moreover, presence of residual silanol groups that interact with the positive charges on peptides increases nonspecific binding, peak tailing and carryover at higher pH. This effect can be minimized by protonation of silanol groups at pH<4 and by chemical derivatisation of residual silanol groups ("end capping") [117]. On the other hand, polymer-based monolith columns are stable over a wide pH range and can withstand pH up to 12 (or even up to 14 with styrene-based monoliths), are less demanding for preparation and exhibit ten times lower carryover of peptides [116]. A lower carryover could be the result of low content of mesopores. However, low content of mesopores results in reduced surface area hence loadability is limited [118], and shrinkage - swelling might occur. Attempt to overcome these drawbacks was made by preparation of an organic-inorganic hybrid silica monoliths [119].

Silica-based capillary columns are superior over particle-based capillary columns regarding their robustness (top of the column can be just cut off if damaged, column permeability does

not change with pressure fluctuation, no frits are required (monolithic interconnected structure is covalently linked to the inner capillary wall) that can be frequently clogged) [19, 52, 100]. Monolithic capillary columns exhibit lower carryover of peptides than particle-packed capillary columns [120]. Peptide carryover was observed even after five isocratic washing cycles with acetonitrile/isopropanol. The surface area and the presence and size of mesopores could be responsible for this effect [120]. If not properly treated carryover can be a source of errors in qualitative and quantitative bottom-up proteomics.

Above mentioned studies describe application of monolithic columns in discovery based bottom-up proteomics. Number of studies applying monoliths in targeted bottom-up proteomic approach is scarce. Targeted approach enables accurate and reproducible quantification of any protein or a set of proteins in any biological sample [5]. However, it requires more time and skills in method design so it is still used less often. Moreover, the number of peptides that could be simultaneously quantified in complex samples by a targeted approach, based on MS acquisition techniques selected/multiple reaction monitoring and parallel reaction monitoring, was limited to around 200 and 600 respectively [121].

Application of DIA on high-resolution MS systems, five years ago, substantially increased the number of peptides that could be simultaneously quantified, and simplified the design of acquisition methods [122]. Recently, a study was published comparing silica-C8 based monolithic trap and particle-based-C18 trap columns using a targeted approach [123]. The study recommend monolithic trap column because it provided significantly reduced peak widths, required fewer connective parts (hence smaller dead volume), provided lower back pressure (enabled fast loading and equilibration), and self-prepared monolithic trap columns provided the luxury of choosing which functional groups to include. The same research group compared commercial analytical monolithic and particle-based capillary columns in target

quantification of cancer cell proteins involved in a metabolic pathway of interest [124]. The authors could not find significant differences, although slight retention time instability was noticed with monolithic columns. Retention time is one of crucial factors for both, targeted and untargeted extraction of quantitative information from data acquired with DIA hence this method requires retention time normalisation.

Currently, the main properties of mass spectrometers like sequencing speed (cycling time – number of spectra per second), ion current (efficiency of ionisation and ion transmission to detector) and resolution of precursor ion isolation are seen as main limiting parameters for development of bottom-up proteomics [90, 125]. Liquid chromatography is not blamed as one of the main obstacles in HTP MS-based proteomics since many problems concerning technical variability and sample consumption are today successfully reduced [90, 125]. Nevertheless, even relaxed from this kind of pressure, further development of LC should be continued since it can provide important improvements such as: strategies for reduction of unspecific binding in affinity chromatography, increased peak capacity, loadability of polymer based monoliths, reproducibility and robustness [19], on-line systems for protease digestion and multidimensional LC, 3D LC systems [3, 108, 126], systems with parallel analysis in second and third dimension to reduce analysis time [19], new ultra-efficient and fast separation 3D LC technologies for microfluidics [127].

4.1.2 IMER in bottom-up HTP MS-based proteomics

Protease digestion of samples in bottom-up proteomics is usually performed overnight. This time consuming step could be shortened by the use of IMER. Monoliths are particularly suitable for immobilization of enzymes (such as proteases) that act on molecules that have low diffusional constants. Different strategies for preparation of monolith IMERs have been

described [118, 128]. On-line sample trypsinization can be performed in seconds using an IMER microreactor [129-131]. Additional, increase of efficacy can be achieved by co-immobilization with Lys-C [132]. With a new monolithic bioreactor with 3D-printed interface stationary phase, high concentration of protease activity can be achieved [133]. This technology for on-line IMER systems enables further size and sample consumption reduction. Proteases that are sensitive to high concentration of organic solvent present in mobile phase can be applied in on-line fashion using valve-switching systems [134]. Efficacy of IMERs in proteolysis can be increased by high temperature, application of ultrasound, microwaves or infrared light [135]). Protease digestion efficacy is one of the most important sources of variation in quantitative proteomics and thus has to be controlled [136]. Application and advantages of IMERs containing different enzymes for HTP analysis of PTMs have been reviewed in more detail by Yamaguchi et al. [135].

4.2 Monoliths in top-down HTP MS-based proteomics

Proteolytic digestion in bottom-up proteomic approach brings certain undesired loss of information originally contained in protein sequence [87]. Top-down approach provides opportunity to access this information using ultrahigh resolution MS systems. Separation of intact proteins from complex samples in top-down proteomic analysis is challenging task due to the wide range of protein sizes, large dynamic range of their expression (over 10 orders of magnitude), heterogeneity and low abundance of certain proteoforms [135]. Monolithic columns are well suited for separation of molecules with low diffusion coefficients such as large proteins, particularly those above 50 kDa. Their large pores (>> 0.2 nm) and surface area allow for adequate loading capacities, and the fast mass transport driven by convection (Cm) grants the attainment of narrow peak. Also, it is possible to apply high flow rates under low back pressure conditions. Reversed phase mode is the most popular LC mode in on-line

protein separations coupled to MS top-down analysis. However, mostly C4-C8 alkyl chains or phenyl are exploited [87].

Performances of PS-DVB-based monolithic columns in separation of intact proteins for top-down analysis on LC-ESI-LTQ-Orbitrap XL MS system were analysed [137]. The authors concluded that the absence of functional groups capable of hydrophilic or ionic interactions (such as silanols present in silica-based materials) in PS-DVB based monoliths facilitates high recoveries and elimination of carryover during intact protein chromatography, hence provides higher sensitivity. Combined with good peak capacity, these properties made PS-DVB-based monolith column superior over silica-based matrices in separation of complex protein mixtures as well as in characterisation of mixtures of high-molecular weight immunoglobulins.

Separation of human 20S proteasome complex into its components was performed on HPLC system with PS-DVB based trapping and analytical columns [138]. This sample of medium complexity was analysed on HPLC-ESI- Q-q-FT-ICR (15 T) SolariX MS system. Columns peak capacity and duty cycle, and mass accuracy and MS resolution of are seen as critical points where further improvement is necessary to enable analysis of high complexity samples [138]. Experimental study described how peak capacity of PS-DVB-based monolith capillary columns in separation of intact proteins can be influenced by different parameters such as flow rate, gradient steepness, temperature, column length, macropore size, and mass loadability [139, 140].

Special methacrylate-ester-based monolithic capillary columns were developed and applied for separation of histones and analysis of their PTMs [141]. Those columns possess advantages in regards to the analysis time (down to 8 min), selectivity and reduced sample requirements. New methacrylate-based monolithic capillary columns for RP-LC have been recently described [142]. They achieved high peak capacity (>1,000), good column-to-

column and run-to-run reproducibility, good loading capacity, high flow rate, long term high thermal stability (could be used at 75 ^{o}C, but protein chemical stability at this temperature should be considered) in separation of intact proteins.

In the recent study the complementarity of shotgun bottom-up and top-down technologies for the qualitative and quantitative analysis of complex proteomes and detection of cancer-specific aberrations at the peptide and proteoform levels was explored [143]. Particle-based and PS-DVB-based monolithic capillary columns were used in top-down approach for analysis of the low molecular weight proteome (<30 kDa) using UHPLC-ESI-LIT-Orbitrap Elite MS system. Top-down approach quantified almost 1,000 proteoforms mapping to 358 proteins. Bottom-up approach identified more than 3 times more identifications, but certain PTMs were accessed exclusively by top-down approach, confirming its importance as complementary techniques, as well as the necessity of mixed bottom-up/top-down approach in analysis of PTMs.

Monolithic capillary columns can be produced in the laboratory without needing to purchase expensive media, packing solvent and high-pressure packing devices. Moreover, production of columns with id smaller than 75 μm is easier than with particles. There are problems with reproducibility of published protocols, but once mastered they are more cost-efficient. Safety issues concerning the use of toxic chemicals are present, while there is no need for the use of high-pressure devices.

Acknowledgment

The authors acknowledge support from Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant No. 172048, 172055 and 172049) and by Program of the European Union FP7 HTP-GlycoMet (contract #324400; author U. Andjelković).

Conflict of Interest Statement

The authors have no financial or commercial conflicts of interest to declare regarding the material presented in this paper.

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Table 1. Some of the literature describing synthesis of the most often used monolithic materials for protein purifications, protein and peptide separations in proteomics.

Type of the monolithic suport		Reference
Silica-based		Minakuchi, H., Nakanishi, K., Soga, N., Ishizuka, N., Tanaka, N., *Anal. Chem.* 1996, 68, 3498-3501.
		Tanaka, N., Kobayashi, H., Nakanishi, K., Minakuchi H., Ishizuka, N., *Anal. Chem.* 2001, 73, 420A–429A.
		Guiochon, G., *J. Chromatogr. A* 2007, 1168, 101–168.
		Nunez, O., Nakanishi, K., Tanaka, N., *J. Chromatogr. A* 2008, 1191, 231-252.
		Tanaka, N., McCalley, D.V., *Anal. Chem.* 2016, 88, 279-298.
		Wu, R., Hu, L., Wang, F., Ye, M., Zou, H., *J. Chromatogr. A* 2008, 1184, 369–392.
Organic polymer-based	Polyacrylamide	Svec, F., *Electrophoresis* 2008, 29, 1593–1603.
		Guiochon, G., *J. Chromatogr. A* 2007, 1168, 101–168.
		Levkin, P.A., Eeltink, S., Stratton, T.R., Brennen, R., Robotti, K., Yin, H., Killeen, K., Svec, F., Fréchet, J.M.J., *J. Chromatogr. A* 2008, 1200, 55-61.
		Eeltink, S., Wouters, S., Dores-Sousa, J.L., Svec, F., *J. Chromatogr. A* 2017, *1498*, 8-21.
		Tanaka, N., McCalley, D.V., *Anal. Chem.* 2016, 88, 279-298.
		Wu, R., Hu, L., Wang, F., Ye, M., Zou, H., *J. Chromatogr. A* 2008, 1184, 369–392.
	Polymethacrylate	Vlakh, E.G., Tennikova, T.B., *J. Sep. Sci.* 2007, 30, 2801-2813.
		Gunasena, D. N., El Rassi, Z., *J. Sep. Sci.* 2011, 34, 2097-2105.
		Guiochon, G., *J. Chromatogr. A* 2007, 1168, 101–168.
		Groarke, R.J., Brabazon, D., *Materials* 2016, 9, 446-479.
		Levkin, P.A., Eeltink, S., Stratton, T.R., Brennen, R., Robotti, K., Yin, H., Killeen, K., Svec, F., Fréchet, J.M.J., *J. Chromatogr. A* 2008, 1200, 55-61.
		Eeltink, S., Wouters, S., Dores-Sousa, J. L., Svec, F., *J. Chromatogr. A* 2017, *1498*, 8-21.
		Tanaka, N., McCalley, D.V., *Anal. Chem.* 2016, 88, 279-298.
		Wu, R., Hu, L., Wang, F., Ye, M., Zou, H., *J. Chromatogr. A* 2008, 1184, 369–392.
	Poly(styrene-co-divinylbenzene)	Wang, Q.C., Svec, F., Fréchet, J.M.J.D20, *Anal. Chem.* 1993, 65, 2243-2248.
		Walsh, Z., Levkin, P.A., Paull, B., Svec, F., Macka, M., *J. Sep. Sci.* 2010, 33, 61–66.
		Guiochon, G., *J. Chromatogr. A* 2007, 1168, 101–168.
		Eeltink, S., Wouters, S., Dores-Sousa, J. L., Svec, F., *J. Chromatogr. A* 2017, *1498*, 8-21.
		Vaast, A., Terryn, H., Svec, F., Eeltink, S., *J. Chromatogr. A* 2014, 1374,

		171–179.
		Tanaka, N., McCalley, D.V., *Anal. Chem.* 2016, 88, 279-298.
		Wu, R., Hu, L., Wang, F., Ye, M., Zou, H., *J. Chromatogr. A* 2008, 1184, 369–392.
Organic polymer-based with nanoparticles	Au	Lv, Y., Maya Alejandro, F., Fréchet , J.M.J., Svec, F., *J. Chromatogr. A* 2012, 1261, 121– 128.
		Liang, Y., Wu, C., Zhao, Q., Wu, Q., Jiang, B., Weng, Y., Liang, Z., Zhang, L., Zhang, Y., *Anal. Chim. Acta* 2015, 900, 83-89.
	Hydroxyapatite	Krenkova, J., Lacher, N.A., Svec, F., *Anal. Chem.* 2010, 82, 8335-8341.
	TiO2	Rainer, M., Sonderegger, H., Bakry, R., Huck, C. W., Morandell, S., Huber, L. A., Gjerde, D. T., Bonn, G. K., *Proteomics* 2008, *8*, 4593-4602.
		Černigoj, U., Gašperšič, J., Fichtenbaum, A., Lendero Krajnc, N., Vidič, J., Mitulović, G., Štrancar, A., *Analy. Chim. Acta* 2016, *942*, 146-154.
Hybride organo-silica-based		Gharbharan, D., Britsch, D., Soto, G., Weed, A.-M. K., Svec, F., Zajickova, Z., *J. Chromatogr. A* 2015, *1408*, 101-107.
		Wu, M., Wu, R., Li, R., Qin, H., Dong, J., Zhang, Z., Zou, H., *Anal. Chem.* 2010, *82*, 5447-5454.
		Liu, S., Peng, J., Liu, Z., Liu, Z., Zhang, H., Wu, R., *Sci. Rep.* 2016, 6, 34718
		Tanaka, N., McCalley, D.V., *Anal. Chem.* 2016, 88, 279-298.

Table 2. Some of the commercially available monolithic materials for protein purifications, protein and peptide separations in proteomics.

Producer or Supplier	Product line	Product	Type of support	Functional group	Formats
BIA Separations	CIMmultus™ Disposable CIM® Tube CIMac™ Analytical	QA - Strong AEX	Poly(glycidyl methacrylate -co-ethylene dimethacrylate) highly porous monolith	Trimethylamine	Columns
		DEAE - Weak AEX		Diethylamine	
		EDA - AEX/Activated		Ethylenediamine	
		SO3 - Strong CEX		Sulphonyl	
		COOH - Weak CEX		Carboxyl	
		OH - HIC		Hydroxyl	
		C4 A - HIC		Low ligand density butyl	
		C4 HLD - HIC		High ligand density butyl	
		IDA - IMAC		Iminodiacetic acid	

		r-Protein A - Affinity		Recombinant protein A	
		r-Protein G - Affinity		Recombinant protein G	
		r-Protein L - Affinity		Recombinant protein L	
		XY - Activated		Epoxy	
		CDI - Activated		Carbonylimidazole	
		HIDA - Activated		Hydraside	
		AE - Activated		Aldehyde	
	Specialised CIMac™ Analytical	AAV full/empty		Trimethylamine	
		Adeno		Trimethylamine	
		pDNA		Diethylamine	
	CIM® enzymatic reactors	Trypsin		Trypsin	
	Custom made			IEX, HILIC, HIC, IMAC, IMER, Affinity, Activated for further derivatisation	Columns, discs, tips, 96-well plate
GL Sciences	MonoCap	MonoCap C18 HighResolution 2000	Silica monolith	C18	Capillary columns
		MonoCap C18 HighResolution Ultra 2000			
		MonoCap HILIC-UP HighResolution 2000		Ureidopropyl groups	
	MonoSpray	MonoSpray C18 Nano		C18	Capillary nano sprayer
	MonoSpin	MonoSpin C18/C18 FF		C18	Spin columns
		MonoSpin Amide		Amide groups	
		MonoSpin CBA		Carboxylic acid groups	
		MonoSpin NH2		Aminopropyl groups	

		MonoSpin SCX		Propyl benzene sulfonic acid groups	
		MonoSpin SAX		Trimethyl aminopropyl groups	
		MonoSpin PBA		bonded with phenylboric acid groups	
		MonoSpin TiO		TiO2	
		MonoSpin Trypsin*		Trypsin	
		MonoSpin C18-CX		C18 and propyl benzene sulfonic acid groups	
		MonoSpin C18-AX		C18 and trimethylaminopropyl groups	
		MonoSpin Ph		Phenyl	
		MonoSpin ME		Iminodiacetic acid	
		MonoSpin Phospholipid		TiO2 and ZrO2	
		MonoSpin ProA		Protein A	
		MonoSpin ProG		Protein G	
	MonoTip	MonoTip C18		C18	Pipette or spin tips
		MonoTip Trypsin		Trypsin	
		MonoTip TiO		Coated with TiO2 nanoparticles	
Isco*	Isco Monolithic Columns*	RP-all	PS/DVB	Phenyl	Columns
		RP-pep			
		SuperSAX	Polymethacrylate	Quaternerary amine (Q)	
		WAX		Tertiary Amine (DEAE)	
		WCX		Carboxylic acid	
LC Packings /Dionex	Swift	IonSwift MAX-100	PS/DVB	Alkanol quaternary ammonium	Columns
		IonSwift MAC (MAC-100, MAC-200)		Alkanol quaternary ammonium	
		PepSwift	Poly(divinylbenzene-	Phenyl	
		ProSwift RP-			

		4H	coethylvinylbenzene-styrene) co-polymer		
		ProSwift RP-1S			
		ProSwift RP-2H			
		ProSwift RP-3U			
		ProSwift SAX-1S	Polymethacrylate	Quaternary amine	
		ProSwift WAX-1S		Tertiary amine	
		ProSwift WCX-1S		Carboxylic acid	
Thermo Scientific	Dionex	Dionex™ IonSwift™ MAX-100 Analytical & Guard Columns	PS/DVB	Alkanol quaternary ammonium ion	Guard columns and Columns
		Dionex™ IonSwift™ MAC Monolith Anion Concentrator Columns (MAC-100, MAC-200) for IC		Alkanol quaternary ammonium ion	
	Swift	DNASwift SAX-1S Columns	Latex Coated Monolith	Quaternary ammonium ion, diethyl methyl amine	Columns
		PepSwift™ Monolithic Capillary LC Columns	Polystyrene copolymer	Phenyl	Capillary columns
		ProSwift™ RP-4H Capillary Monolithic HPLC Columns			
		ProSwift SCX-1S	Polymethacrylate	Sulfonic acid	Columns
		ProSwift ConA-1S Affinity Columns		Concanavalin A	
Merck	Chromolith	RP-18e HPLC Columns	Silica monolith	C18	Columns

		HighResolution RP-18e columns			
		RP-18e Standard HPLC Columns			
		RP-8 Endcapped HPLC Columns		C8	
		Si HPLC Columns			
		NH2 HPLC Columns		Amine	
		SemiPrep HPLC Columns		C18	
		CapRod® HPLC Columns RP-18			
		CapRod® HPLC Columns RP-8		C8	
		RP-18 endcapped guard columns monolithic ready-to-use HPLC column		C18	Guard colum
		CapRod RP-18e Trap			Trap columns
		CapRod RP-8e Trap			
		CapRod RP-18e HR			Columns
		CapRod RP-18e HR			
		Performance Si			
		Performance RP-8e		C8	
		Performance NH2		Amine	
		Performance RP-18e		C18	
		SpeedROD NH2		Amine	
		SpeedROD RP-18e		C18	
		Flash NH2		Amine	
		Flash RP-18e		C18	

		FastGradient RP-18e			
Phenomenex	Onyx	Onyx Monolithic C18	Silica monolith	C18	Columns
		Onyx Monolithic C8		C8	
		Onyx Monolithic HD-C18		C18	
		Onyx™ Monolithic Semi-PREP C18			
		Onyx™ Monolithic C18 Guard Cartridges			Guard colum
		Onyx Monolithic Si		Normal phase	Columns
Agilent	Bio-Monolith	Bio-Monolith Protein A	Poly(glycidyl methacrylate -co-ethylene dimethacrylate) highly porous monolith	Protein A	Columns
		Bio-Monolith Protein G		Protein G	
		Bio-Monolith SO3		Sulfonyl groups	
		Bio-Monolith QA		Trimethylamon groups	
		Bio-Monolith DEAE		Diethylamino groups	
BioRad	UNO	UNO Monolith Anion Exchange Columns Q	Polyacrylamide-based copolymers	Trimethylamon groups	Columns
		UNO Monolith Cation Exchange Columns S		Sulfonyl groups	

Table 3. The applications of monolithic matrices in HTP protein/peptide analysis

Application		Sample	Chromatographic mode, format or method	Reference
High-throughput protein/peptide purification or screening	Fractionation	Complex protein mixtures	IEC, HIC	50, 51, 77, 78, 81-85
		Viruses, cells, exosomes		52, 86
	Enrichment		Affinity capture by Ab	72-76
			96-well plate	75, 76, 79
		Glycoproteins/ glycopeptides	Affinity capture by lectins	60-63, 66
			Borronate-affinity capture	67
			HILIC	23, 68, 69
		HS- containing peptides	Monolith with Au nanoparticles	42
		Biotin labeled proteins/peptides		70
		Phosphopeptides	IMAC	54-56
			Hydroxyapatite	57
			TiO2	58, 59
	Protein depletion	Blood plasma or serum, cell culture medium	Affinity	52, 80
			IEC	81
	Concentration of the sample			98
High-throughput MS-based proteomics	Bottom-up	Complete proteome	One shot RP-LC	94-96, 105, 106, 108, 109, 110, 124
			One shot HILIC	27
			Multidimensional LC	99, 100-102, 112
		Glycoproteome		23, 68
		Phosphoproteome		38, 56
		Other PTMs		115
			IMER	129-131, 135
	Top-down			137, 138, 141