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Review

Recent Strategies for Using Monolithic Materials in Glycoprotein and Glycopeptide Analysis

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Abstract: There is continuous effort towards developing monolithic materials as solid supports for the separation, enrichment, and digestion of glycoproteins. The intention of this review is to discuss and summarize work reported in this area during the period 2015–2021 as a follow-up to our prior review. Reports from the past three decades have already proven the advantages of monolithic materials, such as the ease with which they can be prepared and functionalized, their high permeability and low resistance to mass transfer, and their stability over a wide range of pH. Recent works on glycoprotein analysis introduce different strategies in using monolithic materials specifically in separation, enrichment, and identification of glycoproteins, glycopeptides, and free glycans. A majority of these are focused on boronic acid affinity-based technique and others on lectin affinity and HILIC-based techniques. There are also newly developed ligands that utilize different interactions with glycans, such as encapsulation into β-cyclodextrin vesicles, CH- or OH-π interactions with fullerenes, immunoaffinity with monoclonal antibodies, H-bonding interactions with metallophthalocyanines, coordination interactions with cobalt phthalocyanine tetracarboxylic acid, and hydrophilic interaction with cyclodextrin molecular tubes, zwitterionic iminodiacetic acid, and boric acid. Recent strategies for developing on-line, multidimensional systems use immobilized monolithic enzyme reactors (IMERs) for high-throughput glycoprotein analysis. These works serve as contributions to better understand glycan structure-function relationship, as glycoproteins are now widely accepted disease biomarkers.

Keywords: chromatography; monolith; glycan; glycoprotein; lectin; mass spectrometry

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1. Introduction

A monolith is a single piece of porous rigid material. The word "monolith" comes from the Greek "monolithos" that combines "mono" = single and "lithos" = stone. In separations, a monolith is a continuous microporous separation medium prepared inside a column or tube, in a capillary, confined in a mold, or on the surface of a support [1]. The idea of using a monolithic structure in separations likely started in 1952, when a continuous piece with a porous gel structure was used in the electrokinetic ultrafiltration analysis of polysaccharides. However, the pore structure collapsed upon application of a high enough hydrostatic pressure for the liquid to flow through [2]. Then, in 1967, "a continuous polymer matrix" was used in gel filtration as an alternative to cross-linked polysaccharide-based particles [3]; however, hydrostatic pressure caused failure of the hydrogel structure and column clogging. In the early 1970s, in situ preparation of polymerized foams inside large chromatographic columns was reported [4,5]. Though successful results were obtained, their use was not widely accepted due their poorer performance and features compared to packed columns. The shortcomings included a too complex preparation, lower thermal stability in GC, fixed surface chemistry that limited the separation, and uncertain stability under long-term exposure to solvents and analytes and column-to-column reproducibility. It took almost 20 years until a truly successful monolithic structure was reported, a continuous

polymer bed that was able to withstand hydrodynamic flow at flow rates typically used in LC [6]. The term "monolith" was introduced in a report in 1993 to describing a stationary-phase made of a single piece of functionalized cellulose. The story of reviving monolithic structures is narrated gracefully by Svec in his historical overview of monolithic columns [7]. Different strategies were employed to resolve the shortcomings. Now, monolithic columns are showcasing competitive performance in separations with packed columns. The current monolithic materials are very useful not only on liquid chromatography separations but also in other applications, such as enzyme immobilization, solid phase synthesis, sample preparation, bioengineering, diagnostics, and many more.

This review is the continuation of our previous one published in 2015 in this journal [8] and covers recent strategies introduced for using monolithic materials in IMERs for enzyme digestion and deglycosylation of glycoproteins as stationary phases for separating and enriching glycans, glycopeptides, and glycoproteins and as adsorbents for extracting glycoproteins from biological samples. This review covers 56 new research works from 2015–2021 and also includes background material and discussion of advances in the relevant technologies and materials.

2. Glycoprotein Analysis

The field of glycoprotein analysis benefits from the achievement of new milestones in developing monolithic materials. Glycoprotein analysis uses both glycomics and glycoproteomics approaches (Figure 1). Glycomics studies the structure and biological functions of glycans. Glycans (also called as oligosaccharides) are the sugar units found in glycoproteins and in any form of glycoconjugate. In glycoprotein analysis, these glycans are released from glycoproteins using enzymes. On the other hand, glycoproteomics is the study of protein glycosylation and its influence on protein conformation and biological function. In glycoproteomics, site-specific connectivity between glycans and proteins is identified through glycopeptide analysis. Glycopeptides are produced from digestion of glycoproteins using enzymes.

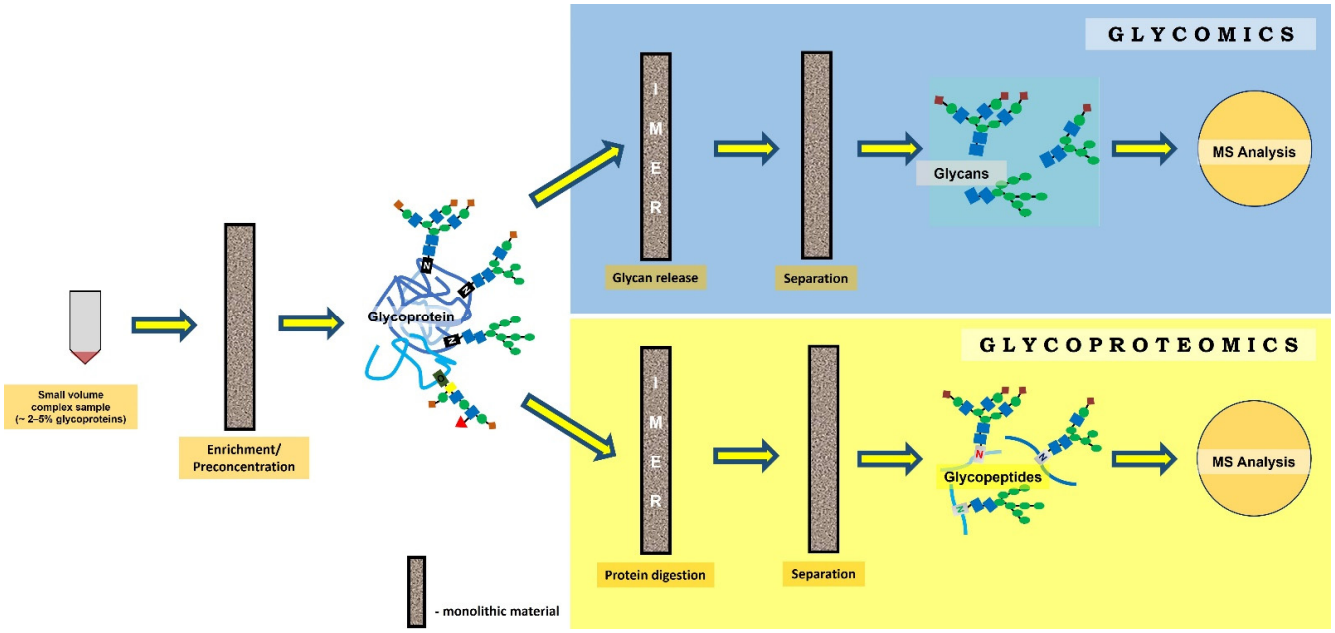

Figure 1. Use of monolithic materials in glycoprotein analysis as solid supports in protein digestion, glycan release, enrichment, and separation processes. The approach in glycoprotein analysis can be either towards glycomics through glycan analysis or towards glycoproteomics through glycopeptide analysis.

A generic workflow in glycoprotein analysis usually starts with the isolation and enrichment of glycoproteins from a complex biological sample. Selected enzymes, such as PNGase F, can then be used to release the glycans from the glycoprotein. The protein may also be enzymatically digested for analysis. A labeling step can be done afterwards to improve detection. The produced glycans, glycopeptides, or deglycosylated peptides then undergo separation by high-performance CE or nano-LC and are then detected using MS [9]. The goals in glycoprotein analysis are to identify the glycosylation sites, elucidate the glycan structures, identify the microheterogeneity of glycans present in a glycopeptide, and to quantify glycan and glycoproteins [10].

Glycosylation is the process wherein glycoproteins are formed by the regulated assembly of sugar moieties to form glycans attached covalently to proteins after translation occurs in the ribosome. It is the most prevalent of the post-translational modifications (PTMs) that proteins undergo. Glycans are found linked to proteins through the amide nitrogen atom on the R-group of an asparagine (Asn) residue found within a consensus sequence of Asn-Xxx-serine(Ser)/threonine(Thr), where Xxx is any amino acid other than proline. This modification is termed *N*-glycosylation, which is the major type of glycosylation [11]. Glycans can also be linked to protein through the hydroxyl groups on the side chain of Ser and Thr residues, and this is referred to as *O*-glycosylation [12]. Glycans attached to proteins are involved in biological events, including cellular surface recognition, cell−matrix interactions, inflammation, tumor immunology, protein folding, and protein stabilization. Abnormal protein glycosylation results in changes in glycoprotein concentrations and alteration of the glycan structure and has been found to play a role in various types of cancer [13]. In the bloodstream, secreted glycoproteins from cells with abnormal glycosylation can reflect an abnormal state of malignant cells. Due to this, numerous glycoproteins are now widely accepted as cancer biomarkers [14].

The structures of glycoproteins and other glycoforms are often heterogeneous due to variations in their compositions, configurations, and linkages [15]. Microheterogeneity of glycan structures is due to glycan branching resulting from overlapping reactions of different enzymes during their synthesis. Glycosylation may also occur at more than one amino acid sequence, and therefore, different glycan structures can be linked to a specific amino acid sequence in a peptide. MS has proven to be a primary method in high-sensitivity and high-throughput glycoproteomics [16,17]. The availability of advanced MS techniques allows characterization of intact glycopeptides. Electrospray ionization (ESI) is the most used technique to ionize glycopeptides during MS analysis. The advantage of ESI ionization is that glycopeptide ions remain intact and are delivered to the mass analyzer without structural decomposition or loss of labile groups [18]. Matrix-assisted laser desorption/ionization (MALDI) is another ionization technique used in the MS analysis of glycopeptides. Unlike ESI ionization, wherein multiply charged glycopeptides are typically formed, singly charged glycopeptide ions are produced through MALDI ionization [19]. This reduces the complexity of MS spectra and a higher signal intensity is achieved since the produced glycopeptides do not split into different charge states.

A challenge in MS analysis is the ambiguity in mass assignment for glycopeptides that have similar m/z values, as occurs when analyzing isobaric glycopeptides that differ in glycan linkages or peptide sequences. Tandem MS analysis is therefore needed to isolate and achieve further fragmentation to obtain detailed structural information [20]. Developments in MS instrumentation have enabled sensitive and high mass accuracy measurements as well as of ion fragmentation techniques that generate more informative tandem mass spectra. A common fragmentation mode routinely used in MS/MS analysis is collision-induced dissociation (CID). CID is a collision-based activation mode that allows for determination of glycan composition and connectivity of monosaccharide units based on the peaks from glycosidic bond cleavages [21]. However, little or no peptide sequence information is available when glycopeptides are subjected to CID. Glycopeptide sequence and glycosylation sites can be determined using a combination of electron capture dissociation (ECD) and electron transfer dissociation (ETD), both of which are two-electron based

fragmentation techniques [22]. Using these techniques, significant peptide dissociation occurs for glycopeptides while preserving the glycan moiety on the peptide. Another fragmentation approach is infrared multiphoton dissociation, in which the glycopeptide precursor is fragmented using infrared photons from CO_2 lasers, increasing its internal energy and causing cleavage of glycosidic bonds [23]. Automated glycoproteomic analysis is a method of choice with the increasing development of software that can interpret glycopeptide mass spectral data [24]. This is very useful when a large amount of data is needed to analyze. Bioinformatics tools now utilize MS and MS/MS CID data to predict glycopeptide compositions [25].

Despite of the latest innovations in MS, glycans remains analytically challenging due mainly to microheterogeneity at their glycosylation site and their relatively low abundance and their poor ionization in MS. Enrichment of glycoproteins or of glycopeptides produced by digestion of glycoproteins, or both, is required during the preparation of samples for analysis.

3. Advances in Monolithic Materials for Separations

The monolithic structures of interest here can largely be classified into two major categories: (1) organic monoliths that are typically composed of synthetic polymers and (2) inorganic monoliths that are mostly based on silica. Organic polymer-based monoliths are usually prepared in situ within a column or mold from liquid precursors, while those that are silica-based are often first prepared and then fit inside a tube to make the column [26]. Both types exhibit a high porosity with pores in low micrometer range that are large enough for efficient flow at a sustainable backpressure. Organic polymer-based monoliths have a heterogeneous structure that resembles an interconnected network of non-porous microglobules with large pores (15–100 nm) and with typical specific surface areas in the low tens of $m^2\ g^{-1}$ [27]. Silica monoliths have a bimodal pore structure with typical specific surface area in the hundreds of $m^2\ g^{-1}$ [28]. Hybrid organic-silica-based monoliths are currently receiving more attention due their potential to combine the advantages of both organic polymer- and silica-based monoliths [29].

There are studies that compare the performances of organic polymer- and silica-based monoliths. These studies show that organic polymer-based monolithic columns are well suited for separation of large molecules but not for the separation of small molecules. In contrast, silica-based monolithic columns enable fast and efficient separations of smaller molecules. Therefore, these two column technologies are complementary [30,31]. Recently, a comparative study was done using commercially available C18 monolithic columns [32]. Three silica-based monolithic columns that differ in length of bonded alkyl chain were used—Chromolith RP-8e, Chromolith RP-18, and Chromolith HR RP-18. These were compared to three polymer-based monolithic columns that differed in pore sizes—ProSwift RP-1S, ProSwift RP-2H, and ProSwift RP-3U. Performance in reverse phase (RP) separations at optimized conditions for all monolithic columns was used for comparison, and silica-based monolithic columns were better able to separate small molecules and acylated peptides. Organic polymer-based monolithic columns were found to separate proteins better at separation times of less than one minute.

In columns, monoliths can be readily prepared using reactions, such as polycondensation [33], polymerization [34], click reaction [35], and other processes. Monolithic columns are characterized by high permeability due to large flow-through pores that allow high pressure. Furthermore, due to this high porosity, resistance to flow is small and allows faster convective flow as compared to the slower diffusion within the pores of particulate packings. Through the years, monolithic columns have been developed in a way that they can achieve comparable performance with the counterpart packed columns. For example, large flow-through pores of monolithic columns allowing high flow rates gave three times faster analysis than the 5-µm particle-packed columns of the same length used at the same operating pressure with comparable separation efficiency [28].

The recent strategies in the development of monolithic materials are focused on improvement of separation efficiency by increasing the capacity, modulation of selectivity, and their novel applications.

3.1. Modifying Polymerization Mixture and Optimizing Polymerization Conditions

The usual strategy to control chromatographic performance of both types of monolithic columns is by tuning structural porosity and surface chemistry of the monolith. This can be done by employing desired preparation conditions. Organic polymer monoliths are usually prepared by free-radical polymerization of a chosen organic monomer [36,37]. Silica-based monolithic columns are usually made using by sol-gel technology [38,39]. To tune the size of through-pores and silica skeletons, the composition of the reaction mixture and concentrations of silane and polyethylene glycol are controlled [40]. To optimize the porosity of polymer monoliths, the relative amounts of porogen and cross-linking monomer in the polymerization mixture, reaction temperature and polymerization time are controlled [41]. In one study, preparation conditions, such as amount of porogen, catalyst concentration, and polymerization temperature and time, were controlled to optimize chromatographic performance of polymerized monolithic columns prepared from 3-(methacryloyloxy)propyltrimethoxysilane under thermal (TSG) conditions [42]. Amounts of co-monomers, such as 1-vinylimidazole (Vim) or 4-vinylpyridine (VP), in the initial polymerization mixture can also be varied to control the column selectivity and permeability [43]. Functionalization of hybrid monoliths can also be done by adding organic functional monomers, such as methacrylates, in the pre-polymerization mixture, and these organic groups (allyl, vinyl, aminopropyl, mercaptopropyl) are then utilized for further surface modification [44].

Varying the PEG concentration in the mixture used for sol-gel synthesis during the preparation of silica-based monoliths can produce skeleton structures with very low capillary-to-domain size-aspect ratios and an increased number of branches and node points. In a monolith structure, an aspect ratio of 2 corresponds to a monolith exhibiting a single node point, which is the furthest reduction in capillary-to-domain size aspect-ratio that can be achieved. The combined skeleton and through-pores constitute the domain size. Silica monolith columns can suffer from severe heterogeneity effects. In particle-packed bed columns, decreasing the capillary diameter could reduce the distance across which the heterogeneity effect has to be relaxed by diffusion and so lower the eddy dispersion. In a study, a TMOS-based (TMOS, tetramethoxysilane) silica monolithic column was prepared with a capillary-to-domain size-aspect ratio of the order of 5:2 [45]. Another strategy is the use of PEG and hydrothermal treatment to produce monolithic silica columns with smaller domain sizes. Smaller domain size in monolithic column reduces the flow resistance and therefore attains better efficiency, i.e., obtains a given number of theoretical plates (N) or peak capacity in the shortest possible time. The use of PEG of molecular weight (MW) = 20,000 produced domain sizes below 2 μm and resulted in plate height value (H) as low as 4 μm for small molecules when applied in the capillary column format [46]. In one study, octadecylsilylated monolithic silica capillary columns (i.d. = 50 and 100 μm) were prepared using PEG with MW = 20,000 and with hydrothermal treatment (at 87 °C) to attain sub-2-μm domain sizes in the mesoporous 12–14-nm range [47]. Based on these chromatographic parameters, the prepared monolithic column outperformed the commercially available 2-μm particle-packed bed capillary column.

Using polyhedral oligomeric silsesquioxane (POSS)-based monomer in preparation of hybrid polymer monolithic column is a strategy for enhancing the mechanical and pH stability of the monolith. POSS reinforces the polymeric networks at the molecular level resulting in development of ceramic-like properties that enhance the mechanical and pH stability of the monolith. Polyhedral oligomeric vinylsilsesquioxanes (vinyl POSS) were used in one study to prepare a hybrid polymer monolithic column via radical-mediated step-growth thiol-ene chemistry [48]. Two thiol linkers, pentaerythritol tetra(3-mercaptopropionate) (PETMP) and 2,2′-(ethylenedioxy)diethanethiol (EDDT), were used, and it was found that

the choice of thiol-linker is a factor in attaining near-ideal nanoscale networks with vinyl POSS. In another study, acrylopropyl polyhedral oligomeric silsesquioxane (acryl-POSS) was used in a "one-pot" approach via photoinitiated thiol−acrylate polymerization. The preparation was very fast (5 min) and used monothiol monomer (1-octadecanethiol or sodium 3-mercapto-1-propanesulfonate) in UV-transparent fused-silica capillaries [49].

Incorporation of nanomaterials into capillary monolithic columns for separation of small molecules, peptides, and proteins is one strategy to achieve good selectivity and/or high performance for enrichment and/or separation [50]. Examples of these nanomaterials are carbon nanotubes (CNT), graphene nanosheets, fullerenes, hydroxyapatite nanoparticles, and gold nanoparticles. These nanomaterials are incorporated into monoliths via post-modification and "one-pot" methods. In one study, mesoporous carbon nanomaterial (MCN)-based butyl-silica capillary hybrid monolithic column (MCN-C4-monolith) was prepared via crosslinking of (3-aminopropyl)trimethoxysilane (APTMS)-modified MCNs (AP-MCNs) with tetramethoxysilane (TMOS) and n-butyltrimethoxysilane (C4-TriMOS) [51]. By using this monolithic column, cLC separation of alkylbenzenes and proteins showed increased reversed-phase retention as compared to butyl-silica monolith without AP-MCN. This developed monolithic column is promising in bottom-up proteomics. Another reported strategy is encapsulating trimethylated-β-cyclodextrin (TM-β-CD) into polymer monolithic capillary columns to achieve good enantioseparation efficiency of monolithic columns. Cyclodextrins (CD) and their derivatives are used as chiral selectors in enantioselective separation of racemates. Differentiation between enantiomers of chiral pharmaceuticals is important, as any discrepancy in their 3D structures could lead to different pharmacological and toxicological properties. The TM-β-CD-encapsulated monolithic capillary column was prepared by one-pot in situ copolymerization of ethylene glycol dimethacrylate (EDMA), glycidyl methacrylate (GMA) monomers, and 1-propanol, 1,4-butanediol as progenic solvents in presence of TM-β-CD solution within fused silica capillaries (150 µm i.d.) [52]. Incorporation of higher concentration of TM-β-CD resulted to relatively higher surface area, smaller pore size, and higher total pore volume. The formation of host−guest inclusion complexes in the CD cavity is the main mechanism of retention and enantioseparation under reversed-phase conditions [53].

3.2. Gradient Stationary Phase on Monolithic Columns

Creating gradient stationary phases is a proposed strategy to obtain novel selectivity [54]. Separation of analytes in LC is dictated by the selective interactions of the analytes with the stationary and mobile phases. This selectivity could be improved by varying the chemistry of the stationary phase and the conditions of the mobile phase, such as solvent composition and pH. Improved selectivity results in better resolution, which is a challenge in the analysis of complex biological and environmental samples. Polymeric monolithic columns are known for their versatility, ease of preparation, and surface modification. These characteristics are gateways to creating multifunctional columns, i.e., different functionalities along the column or gradient functionality on the surface. In a gradient stationary phase, there is a gradual change, i.e., change in chemical and physical properties, such as charge, hydrophobicity, etc., or in the surface coverage of a specific chemical functionality along the column. One preparation of a continuous gradient stationary phase was achieved using in-house synthesized monolithic silica columns via the controlled rate infusion (CRI) method [55]. Pre-hydrolyzed aminoalkoxysilane solution was infused through the silica monoliths so it could react with surface Si-OH groups. Different selectivity was obtained using this gradient stationary phase when compared to use of uniformly modified and unmodified silica columns. Another method used to create gradient stationary phases is photo-grafting, i.e., sequential, stepwise grafting initiated and controlled by UV irradiation. The density of the graft is proportional directly to the increase in energy applied along the column [56]. A photo-initiated graft procedure was carried out on the surface of monoliths of poly(BuMA-co-EDMA) [57]. Sulfopropyl methacrylate monomer was grafted to produce a gradient functionalized cationic surface. The grafting procedure was optimized using

scanning capacitively coupled contactless conductivity detection (sC^4D) [58]. Gradient stationary phase showed improved resolution when compared to the homogeneously grafted (isotropic) column. There was no change in monolith morphology at the micrometer level after the gradient column was fabricated as observed under SEM.

3.3. Monoliths in Nano-LC Columns and Microfluidic Platforms

Development of a nano-LC column is one of the best strategies for higher separation efficiency and also for the improved profiling sensitivity in LC-MS-based proteomics. A high-efficiency, ultra-narrow bore monolithic LC column (20 µm ID) was coupled to MS by a high-field asymmetric waveform ion mobility spectrometry (FAIMS) Pro interface operated in a 1-h gradient at ultralow flow (ULF) rates (12 nL/min) [59]. ULF LC-MS was proven to increase the depth and sensitivity of proteomic profiling of clinical and biological samples in very limited amounts (low-ng) [60]. The FAIMS Pro interface focuses and separates multiply charged peptide ions from interfering singly charged background ions and thus decreases the level of noise [61].

Integration of miniaturized functions into single microfluidic devices are now garnering attention in process development. For example, microflow reactors and separation columns are being combined in continuous flow micro-systems. Chip electrochromatography (ChEC) is a non-pressure-driven flow chromatographic type of separation, wherein electroosmotic flow (EOF) provides the pumping throughout the interface of the chip device to HPLC tubing. A porous polymer monolithic column was used as a stationary phase in electrochromatographic separation integrated with chemical reactions and electrospray ionization in a chip-based microfluidic system [62]. These connected functions in the microfluidic device were followed by downstream integrated LC-MS. This approach was successfully employed in simple enzymatic conversions and in synthesis of 2-amino-4-phenylthiazole from thiourea and 2-bromoacetophenone as starting materials. A new microfluidic platform was developed by integrating the reversed-phase chromatography using poly-allyl phenoxyacetate (AP) monolith, IMER, and a lysine-glycine-glycine (KGG) imprinted monolith to do on-line protein fractionation, denaturation, digestion, and peptide enrichment [63]. The AP monolith interacts with proteins through hydrophobic interactions, π-π stacking interactions, and other intermolecular forces and has a high degree of protein selectivity. Molecularly imprinted polymers (MIP) can specifically recognize with high sensitivity their imprinted target compounds and template analogs. Integrated to IMER protein digestion, the multi-dimensional microfluidic system allowed simultaneous collection of proteomics and post-translational modification proteomics information.

In one study, three different geometries of chromatographic monolithic columns were designed to study the effect of column 3D geometry, i.e., coiling on liquid chromatographic efficiency [64]. Poly(BuMA-co-EDMA) monolithic columns were prepared by in-column thermal polymerization and were 3D printed using selective laser melting (SLM) in titanium. Different geometries of these columns were 2D (planar) serpentine, 3D spiral, and 3D serpentine. The 2D (planar) serpentine column was a predominantly straight column with a small number of low-aspect-ratio turns. The 3D spiral column had medium-aspect-ratio turns. The 3D serpentine column had high number of repeating high-aspect-ratio turns. All columns were of equal length and i.d., and 3D serpentine chromatographic column obtained higher efficiency according to Van Deemter plots, i.e., aspect-ratio turns at higher linear velocities and shorter analysis times compared to the other columns. Moreover, using 3D serpentine column in isocratic RPLC separations resulted in an average 23% and 245% increase in the number of theoretical plates as compared to the 3D spiral and 2D serpentine columns, respectively. In gradient RPLC separations, it was found that using a 3D serpentine column gave an average increase of 15% in peak capacity compared to the 3D spiral column and an 82% increase compared to the 2D serpentine column. When the 3D serpentine column was used at a faster flow rate, a 58% reduction in the time required for the isocratic separations of the small molecules was found compared to the 3D spiral

column. A 74% increase in peak capacity was found for the gradient separations of proteins using the 3D serpentine column.

4. Recent Strategies Using Monolithic Materials in the Separation and Enrichment of Glycoproteins and Glycopeptides

To improve detection of glycans, a range of enrichment techniques targeting mainly proteolytically digested glycoproteins have been developed. Lectin-specific capturing techniques are capable of enriching a specific glycoform that contains glycan motifs specifically binding with the lectin used. Although this method can be highly selective, the selectivity is limited to a specific glycan structure. The use of multi-lectin columns allows multi-specific enrichment [65]. Lectin-specific approaches can also be integrated with microarray technology for a semi-automated high-throughput analysis [66]. The HILIC technique uses a hydrophilic stationary phase that strongly retains glycans due to their hydrophilicity and separates them from non-glycosylated peptides [67]. This technique is less biased for specific glycan species, and its glycopeptide separation depends largely on the hydrophilicity and size of the glycan moiety. Boronic acid affinity-based techniques selectively target the *cis*-diol groups in glycans to form covalent heterocyclic diesters that can be reversed under acidic pH conditions. However, while it is not promising for selective capturing of glycoforms having a specific glycan structure, it instead can capture a more diverse pool of glycans. The hydrazide-capturing technique also selectively targets the *cis*-diol groups in glycans. *Cis*-diols are oxidized into aldehydes, then covalently coupled with hydrazide immobilized to solid support [68]. The coupling is irreversible, and the bound glycan portion is cleaved using glycosidases (PNGase F) or by chemical derivatization. The nonglycosylated or unbound peptides are removed by washing. Bound glycopeptides are then released by PNGase F for MS analysis. The hydrazide method is time consuming, and the structural information for intact glycan structures on glycoproteins or glycopeptides is not made available. It is also not promising for selective capture of glycoforms having a specific glycan structure.

Among these enrichment techniques, boronic acid affinity-based is receiving much interest due to its unique affinity for *cis*-diol-containing compounds, such as glycopeptides, glycoproteins, and glycans (Table 1).

Table 1. Recent strategies in preparation of monolithic materials for the separation of glycans.

Strategies	Monolithic Materials	New Application to Separations from the Work	Ref/Year
Lectin affinity-based monolithic materials			
Use of an organic support polyvinyl alcohol (PVA-GA) that can participate in many reactions favoring their activation	*Concanavalin A* (ConA) on PVA-GA monolithic column	Addition to the list of supports for lectin immobilization. Eluted by a minimum concentration of 0.6 M glucose solution	[69] 2016
Functionalization with succinimide groups on monolith surface for grafting of lectins via lysine amino groups.	*Lens culinaris agglutinin* (LCA), Con A, and *Ricinus Communis Agglutinin* (RCA) on *N*-acryloxysuccinimide monolith (NASM) column	A method to immobilize multiple lectins that could capture a wide range of glycoproteins/glycoforms in human serum for analysis via LC-MS/MS	[70] 2017
Spin columns for spin column lectin chromatography using a highly hydrophilic (meth)acrylate-based monolithic cryogel	Con A on poly(HEMA-*co*-PEGDA) monolithic cryogel	Good efficiency and selectivity of lectin-modified cryogel towards glycoprotein mixture using MALDI-MS analysis. Spin column was good to use up to the fifth time with no observable loss of affinity.	[71] 2015
Lectin microcolumns for high-performance affinity chromatography (HPAC).	Con A and *Aleuria Aurantia* lectin (AAL) on HPLC-grade porous silica (NUCLEOSIL®)	Low non-specific binding and fast analysis time. Can integrate with on-line detectors or with other columns to create multi-dimensional systems.	[72] 2019
HILIC-based monolithic materials			
Using HALO® penta-HILIC column that contains five OH groups in tandem with mass spectrometric detection	HALO® penta-HILIC column with five OH groups on the bonded ligand	Different selectivity, i.e., retention of glycopeptides increases with the number of monosaccharide units in the glycan moiety	[73] 2018
Tip technology using commercially available extraction tips (StageTip by Thermo Scientific)	Piperazine-modified polymeric monolithic tip	Low cost, yet rapid separation (2 min) due to high selectivity, strong hydrophilicity, high sensitivity, good recovery, and batch-to-batch reproducibility	[74] 2020

Table 1. *Cont.*

Strategies	Monolithic Materials	New Application to Separations from the Work	Ref/Year
Incorporation of fumed silica nanoparticle (FSNPs) and cyano-modified FNSPs (CN-FSNPs) as "stationary phases" onto monolith	Cyano-modified-FSNPs-poly(GMM-co-EDMA) monolith	High selectivity and increased retention. Rapid, low cost, requiring smaller quantities of sample. Microscale analysis of complex biological fluids done efficiently.	[75] 2020
Electrostatic repulsion hydrophilic interaction liquid chromatography using strong anion exchange solid-phase extraction (SAX-ERLIC)	SOLA SAX SPE cartridges (ThermoFisher Scientific)	Identified unique glycopeptides using an LTQ-Orbitrap Elite mass spectrometer that yielded 191 unique glycoforms across 72 glycosylation sites from 48 glycoproteins	[76] 2017
Boronic acid affinity-based monolithic materials			
Use of linear macromolecule porogen (polystyrene)	Poly(VPBA-co-EDMA) monolithic column	Avoided the coarsening of monolithic structure that could result in heterogeneous microporous structures consisting of micron size globular particles; separation of *cis*-diol flavonoid glycosides isomers—isoquercitrin (ISO) and hyperoside (HYP)	[77] 2016
Use of hydrophilic macromonomer oligo (ethylene glycol) methyl ether methacrylate (OEG) was mixed with 3-(acrylamido)-phenylboronic acid (AAPBA) as functional monomer	Poly(AAPBA-co-OEG-co-EDMA) monolithic column	Improved affinity and so improved recovery of HRP (97.51%) and OVA (93.97%) in polymer monolith microextraction (PMME) using the prepared OEG boronate monolith as compared to OEG-free boronate monolith (increase of 30%)	[78] 2018
Use of hydrophilic 4-vinylphenylboronic acid in preparation of hybrid monolith via a simple and convenient "one-pot"	VBPA-silica hybrid monolithic column	Produced mixed-interaction monolith—hydrophilic, cation exchange, and boronic acid affinity; binding pH was as low as pH 7.5.	[79] 2018
Incorporation of nanomaterial graphene oxide into monolithic column	Poly(VPBA-EDGMA-GO) monolith in a PEEK tube	Increased the effective surface area and so improved the extraction efficiency for HRP in an online SPME-HPLC system	[80] 2018
Incorporation of fumed silica nanoparticles (FSNPs) into hybrid monolithic column	Poly(HPMA-Cl-MFSNP-EDMA) monolithic column	Ready access to various functionalities; large surface area. Good separation of alkylbenzenes in nano-liquid chromatography.	[81] 2016
Use of boronic acid functional ligand with lower pKa (3,5-difluoro-4-formyl-phenylboronic acid, pKa = 6.5)	Boronate-silica affinity monolith with 3,5-Difluoro-4-formyl-phenylboronic acid	Higher binding affinity; able to bind to cis-diol nucleoside at physiological condition (pH = 6.5)	[82] 2019
Use of organic-inorganic hybrid monomers, such as 3-aminopropyltriethoxysilane-methacrylic acid (APTES-MAA) and polyhedral oligomeric silsesquioxanes (POSS)	APTES-MAA/POSS-boronate affinity monolith	Good affinity and selectivity for glycoproteins (OVA, transferrin (Trf), HRP), good solvent resistance and pH stability, greater rigidity, and binds to glycoproteins at wide range of pH (5–8).	[83] 2019
Use of organic-inorganic hybrid polyhedral oligomeric silsesquioxane-methacryloyl histidine (POSS-MAH)	(POSS-MAH-PBA) monolithic column	6-fold to 7-fold increase in adsorption capacity; 4.25 times more selective for adenosine and 48.9-fold higher enrichment factor than POSS-MAH free	[84] 2021
Use of molecularly imprinting polymers (MIPs) technology with pseudo-template and surface imprinting to avoid template leakage	Boronate affinity-based surface molecularly imprinted monolith (BA-SMIM)	Homogeneous and excellent imprinted recognition sites that could bind two *cis*-diols; reduced the capturing pH due to nanoconfinement effect of imprinting cavity	[85] 2019
Miniaturization of boronate affinity monolithic column and in-line coupling with capillary zone electrophoresis	AAPBA-functionalized silica monolith	Allowed fully automated system that includes in-line preconcentration/purification, separation, and detection for analysis of *cis*-diols in complex sample; required low sample volume (less than 2 μL) and improved limits of detection (LOD)	[86] 2017
In-line coupling with nano-LC reversed-phase separation	AAPBA-functionalized silica monolith	4-fold increase in the number of phenylboronate sites. Allowed integration of preconcentration and separation steps	[87] 2019
Using a crosslinked polyvinyl alcohol to decorate boronic acid into a microporous polymer structure	Macroporous polymer with polyvinyl alcohol as crosslinker (MP-VPA) matrix	Created hydrophilic boronate affinity matrix that is non-swellable and highly crosslinked	[88] 2016
Incorporating boronic acid monolith in an interface-free multidimensional separation system	Coupled thiol graphene (TG) doped poly(ionic liquid ($ViOcIm^+Cl^-$)) boronate affinity monolith to poly(guanidinium ionic liquid) monolith	Interface-free multidimensional separation system avoids dead volume along the coupled materials. High separation efficiency was attained using CEC in isolating glycoproteins from other non-glycoproteins.	[89] 2015

Table 1. *Cont.*

Strategies	Monolithic Materials	New Application to Separations from the Work	Ref/Year
New strategies for separation and enrichment of glycans that use monolithic materials			
Use of β-Cyclodextrin vesicles (CDVs) to create a pH-responsive monolith	Mesoporous poly(glycidyl methacrylate-pentaerythritol triacrylate) (poly- (GMA−PETA)) monolith grafted with CDVs	15 glycopeptides from Myo digest were captured via controllable enrichment combined with MALDI-MS with limit of detection of 0.1 fmol. 166 intact glycopeptides from 130 glycoproteins in human blood samples were identified.	[90,91] 2018
Use of cyclodextrin molecular tube functionalized with glutamate (gluCDMT)	Poly(HEMA-PETA-gluCDMT)	High binding capacity (~50 mg g^{-1}) and captured glycopeptides (23 HRP glycopeptides and 28 IgG glycopeptides). Good selectivity in HRP/BSA mixture (1:10,000)	[92] 2018
Use of fullerenes bound silica monolithic capillary and a thermo-reactive agent, perfluorophenyl azide	C_{60}- and C_{70}-fullerene bonded columns	Separate 2-aminobenzamide-labeled glucose homopolymers from non-labeled glucose homopolymers by LC under aqueous conditions. Retention rates of disaccharides, such as maltose, trehalose, and sucrose, were determined using C60 column	[93] 2020
Use of monoclonal anti-human fibrinogen antibodies to prepare customized chromatographic monolithic column	Convective interaction media (CIM) monolithic support with immobilized monoclonal anti-human fibrinogen antibodies	Fast and simple immunoaffinity purification of fibrinogen (FIB) from human blood samples	[94] 2017
Use of amorphous TiO_2 modified with boric acid	Monolithic borated titania	Enhanced hydrophilicity and therefore selectivity of towards glycoproteins; binding capacities were 9.3, 26.0, and 53.0 mg g^{-1} for ribonuclease B, HRP, and OVA, respectively	[95] 2018
Use of cobalt phthalocyanine tetracarboxylic acid (CoPcTc)	Poly(GMA-EDMA) monolith grafted with CoPcTc via condensation acylation of carboxyl groups with amine groups	28 IgG and 17 HRP glycopeptides were identified in polymer monolithic microextraction (PMME) coupled with MALDI–TOF MS with high enrichment selectivity	[96] 2018
Use of copper tetra(N-carbonylacrylic) aminephthalocyanine (CuMPc) and iminodiacetic acid (IDA)	Poly(GMA-EDMA-CuMPc-IDA) monolith	Captured and identified a total of 24 IgG glycopeptides and with a detection limit of 5 fmol; high selectivity in a mixture of IgG digest and BSA (1:100 m/m)	[97] 2019
Use of copper tetra(N-carbonylacrylic) aminephthalocyanine (CuMPc) and iminodiacetic acid (IDA)	Poly(GMA-EDMA-CuMPc-IDA) monolith	Captured and identified a total of 20 HRP glycopeptides and with a detection limit of 0.5 fmol μL^{-1}; high selectivity in a mixture of BSA and HRP digests (200:1, m/m)	[98] 2018

4.1. Lectin Affinity-Based Monolithic Materials

Lectin affinity-based methods have been widely accepted in separation of glycans. In fact, there are a number of lectin-based monolithic columns that are now commercially available. Concanavalin A (ConA) HPLC column (ProSwift® ConA-1S monolith column by Thermo Scientific, Waltham, MA, USA) was used to design a protocol using lectin HPLC-GE-MALDI-TOF MS/MS that includes gel electrophoresis (GE) separation, MS detection, and consequential bioinformatic analysis to screen total N-glycoprotein "pool" in cereals [99]. The analysis was also done on water-soluble glycoproteins from wheat durum (*Triticum durum*), barley (*Hordeum vulgare*), and spelt (*Triticum spelta*). The developed method identified low-molecular-weight glycoproteins in the 12–16 kDa range, which belong to alpha amylase/trypsin inhibitors family and are confirmed allergens in wheat and barley flour.

One recent strategy in lectin-based affinity monolithic material is the use of a polyvinyl alcohol—glutaraldehyde (PVA-GA)—interpenetrated network to immobilize Con A and create affinity chromatography column matrix for capturing fetuin [69]. Organic supports, such as PVA, are now used for immobilization of proteins due to their versatility to participate in different reactions that eases their activation [70]. The developed PVA-GA-ConA column achieved a yield of 30% Con A immobilization, and 0.6 M glucose solution was the minimum concentration required to elute fetuin from the column.

Post-polymerization modification (PPM) on monolithic precursors to graft lectins on the surface of monolithic materials is one strategy used to create lectin affinity-based monolithic materials. Different lectins (LCA, Con A, and RCA) were immobilized onto

N-acryloxysuccinimide and ethylene glycol dimethacrylate (poly(NAS-co-EDMA)) monolith [70]. The preparation of the monolith was done via in situ polymerization of *N*-acryloxysuccinimide and ethylene glycol dimethacrylate in a narrow bore stainless steel (NASM) column (1 mm i.d.). NASM has succinimide groups on the surface that can easily bind to compounds with primary amines and with lysine residues in proteins. NASM-lectin columns were able to capture a wide range of glycoproteins/glycoforms from human serum for analysis via LC-MS/MS. The NASM was a useful precursor for PPM not only for lectins but also for octadecyl ligands to create a reversed-phase chromatography (RPC) monolithic column and trypsin to create an immobilized enzyme reactor (IMER).

A highly hydrophilic (meth)acrylate-based monolithic cryogel was activated by epichlorohydrin, immobilized with concanavalin A, and placed in the centrifugal filter device and utilized for spin column lectin chromatography [71]. Macroporous cryogel is a unique hydrophilic monolithic material formed by freezing the initial solution containing monomers, crosslinkers, free radical initiators, and porogens. The (meth)acrylate-based monolithic cryogel was prepared and optimized to have bimodal pore size distribution with flow through macropores of size range 20–50 µm and submicrometer pores in the polymer walls. Polymerization utilized hydroxyethyl methacrylate (HEMA) as monomer, poly(ethyleneglycol) diacrylate (PEGDA) as crosslinker, and TEMED as accelerator. The good efficiency and selectivity of lectin-modified cryogel towards glycoprotein mixture was observed using MALDI-MS analysis. For example, non-glycoprotein BSA in the mixture was not detected in the elution fraction. The prepared spin columns were still good to use up to the fifth time with no observable loss of affinity.

Developing and optimizing lectin microcolumns based on HPLC-grade porous silica (NUCLEOSIL®) is one strategy for high-performance affinity chromatography (HPAC). In HPAC, binding agents, such as lectins, are immobilized onto small, rigid supports to create microcolumns that can withstand the high flow rates and operating pressures of HPLC. These microcolumns require immobilization of a small quantity of ligand and can be used with small sample sizes. Moreover, these microcolumns offer low non-specific binding and fast analysis time and can do so with on-line detectors or with other columns to create multi-dimensional systems. *Concanavalin A* (Con A) and *Aleuria Aurantia* lectin (AAL) were immobilized by reductive amination and packed into 2.1 mm i.d. × 5.0 cm microcolumns [72]. These were used in analysis of glycoform fractions from alpha1-acid glycoprotein (AGP) based on both the degree of branching and level of fucosylation. Separation of non-retained from retained AGP glycoforms using Con A microcolumn (flow rate of 50 µL min^{-1} at 50 °C) was within 20 min or less. Separation using AAL microcolumn (flow rate of 0.75 mL min^{-1} at 50 °C) was within 6 min or less.

4.2. Hydrophilic Interaction Liquid Chromatography (HILIC)

Hydrophilic interaction liquid chromatography (HILIC) is currently gaining attention for separation of polar compounds. First introduced by Alpert in 1990 [67], HILIC elutes polar analytes after their adsorption onto a moderately polar stationary phase using a relatively nonpolar mobile phase. Retention of analytes to the stationary phase involves several intermolecular forces, such as hydrogen bonding, electrostatic interactions, dipole-dipole interactions, and weak hydrophobic interactions, eventually causing the separation [100]. Both silica-based monoliths [101] and organic polymer monoliths [102] with surface-bound polar functional groups were developed as HILIC stationary phases. The micro- and macro-heterogeneity of glycoproteins is one of the main challenges in its analysis. Comprehensive characterization of isomeric glycopeptide structures is difficult in the positive ionization mode of MS due to similar fragmentation patterns produced by collision-induced dissociation. Reverse-phase (RP) chromatography cannot resolve isobaric glycoforms of glycopeptides, but HILIC can. HILIC can separate N-glycan isomers or those that differ only in branching and/or linkage position, derivatized or not.

One major advantage of the HILIC-based technique is its capability to extensively enrich glycopeptides due to its affinity for different glycan types. Due to this, ZIC-HILIC,

a zwitterionic type of HILIC, which is largely dependent on the solvent [103], was used to assess efficiency and selectivity of different mobile phases on glycopeptide enrichment using a method called "Drop-HILIC." Acetonitrile, methanol, ethanol, and isopropanol are four commonly used MS compatible solvents and were tested by spiking synthetic glycopeptides into different concentrations of tryptic BSA peptides, standard glycoproteins, and a complex sample derived from human (depleted and non-depleted) serum. Results showed that acetonitrile was the best overall choice for the retention of both hydrophilic and hydrophobic glycopeptides, while methanol was found unsuitable. For some hydrophobic glycopeptides, using isopropanol gave the best enrichment.

HILIC was also used to assess the performances of different labeling compounds in enhancing the sensitivity of N-glycans using HILIC solid-phase extraction and analyzed using HILIC-UPLC-FLR-MS [104]. The retention of glycan derivatives is highly dependent on the properties of the labeling groups. For example, in reversed-phase liquid chromatography (RPLC), the hydrophobicity of the labeling groups affects the retention and resolution of the glycan derivatives. Free glycans are derivatized to enable their optical detection in LC profiling or to improve their ionization efficiency in MS detection. Three labeling compounds (2-aminobenzamide (2-AB), procainamide (ProA), and *Rapi*FLour-MS (RF-MS) fluorescent tag) were evaluated. N-glycans were first released from IgGs using PNGase F, fluorescently labeled, purified using HILIC solid-phase extraction, and analyzed using HILIC-UPLC-FLR-MS. Results suggest the use of ProA and RF-MS when higher sensitivity is required in the analysis. 2-AB exhibited the lowest sensitivity. All three labeling procedures showed good and comparable repeatability and very similar labeling efficiency. In another work, the effect of fluorescent tags (2-aminopyridine, 2-aminobenzoic acid, 2-aminobenzamide, and ethyl 4-aminobenzoate) on the structure and retention of glycans was investigated [105]. A capillary-packed monolithic ODS phase without end-capping treatment and an amino- and amide-bonded hydrophilic (AAH) phase were developed. In the HILIC separation mode, the AAH column exhibited better resolution for isomaltooligosaccharides than was obtained on a conventional amide column. The cationic amino groups of the AAH column enhanced the resolution of sialylated complex glycans that could not be separated on the conventional amide HILIC column. Using a capillary-packed monolithic ODS column, good resolution for neutral glycans was obtained unlike when using a conventional packed ODS column.

One strategy in HILIC separation is using a superficially porous particle HALO® penta-HILIC column that contains five OH groups on the bonded ligand in analysis of N-glycopeptides of hemopexin [73]. This HILIC technique separated glycoforms of the same peptide backbone, including the separation of isobaric glycoforms in tandem with mass spectrometric detection. The stationary phase showed different selectivity. Increasing the number of monosaccharide units in the glycan increased the retention of the glycopeptides. The presence of sialic acid on glycan structure also increased the glycopeptide retention.

Another strategy is the use of commercially available extraction tips (StageTip by Thermo Scientific, Waltham, MA, USA) to prepare piperazine-modified polymeric monolithic tips via free radical polymerization [74]. The prepared tips were used to enrich glycopeptides from chicken avidin, horseradish peroxidase (HRP), and immunoglobulin G (IgG). A separation time of 2 min was achieved under optimized conditions using 20 cycles per loading, incubation, washing, and elution step, then followed by MALDI-MS analysis (Figure 2). Due to strong hydrophilicity and the accessible binding sites for interaction, high selectivity (1:400 horseradish peroxidase/bovine serum albumin), high sensitivity (100 attomoles), good recovery (89.51%), and batch-to-batch reproducibility (RSD > 1) through HILIC mechanism were attained in glycopeptide enrichment. This tip methodology is rapid and inexpensive, requiring smaller amounts of sample, and is efficient for the microscale analysis of complex biological fluids.

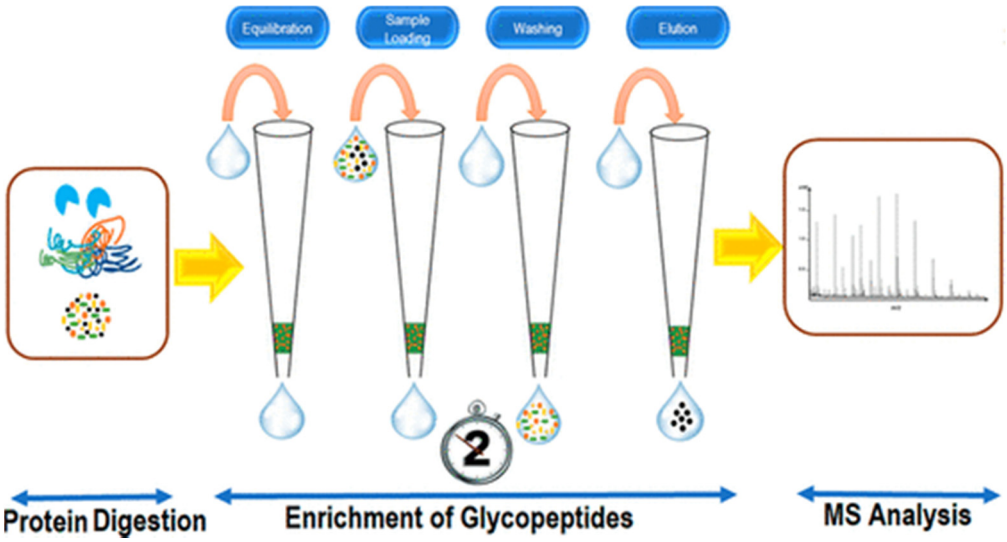

Figure 2. A tip-based strategy using a piperazine-modified polymeric monolithic tip for fast separation (2 min) of glycopeptides and glycans under optimized conditions with 20 cycles per step of loading, incubation, washing, and elution, followed by MALDI-MS analysis. Reprinted with permission from reference [74].

Using organic polymer monoliths in HILIC separations is the current strategy to resolve the lack of availability of polar monomers and the low solubility of the available monomers in organic porogens. Monoliths are subjected to post hydrophilization or the covalent attachment of polar molecules [106] or grafting of allyl groups to where polar groups can be attached [107]. Another way is incorporating fumed silica nanoparticles (FSNPs) [108]. SNPs can be easily modified with different functionalities via their surface silanol groups. Bare fumed silica nanoparticle (FSNPs) and cyano-modified FNSPs (CN-FSNPs) were incorporated to serve as "stationary phases" onto poly(GMM-co-EDMA) monolith as base support [75]. The monolithic column was prepared via in situ polymerization of glyceryl monomethacrylate (GMM) and ethylene glycol dimethacrylate (EDMA). The modified monolithic column was used in hydrophilic interaction liquid chromatography (HILIC) of neutral, polar, and low-molecular-weight solutes. High selectivity factors were observed during the separation. CN-FSNPs-poly(GMM-co-EDMA) monolith gave increased retention of analytes as compared to unmodified FSNPs.

Electrostatic repulsion hydrophilic interaction liquid chromatography strong anion exchange solid-phase extraction (SAX-ERLIC) was used in a site-specific N-linked glycoproteomic analysis in real, complex samples (serum or plasma) using an LTQ-Orbitrap Elite mass spectrometer [76]. SAX-ERLIC was the most robust method when compared with lectin affinity chromatography and Sepharose-HILIC. This allowed SAX-ERLIC enrichment, yielding 191 unique glycoforms across 72 glycosylation sites from 48 glycoproteins, a 100% increase compared to other two enrichment techniques. Moreover, using SAX-ERLIC enrichment had no apparent bias toward specific glycan types.

4.3. Boronic Acid Affinity-Based Monolithic Materials

Preparation of a boronate affinity monolithic column was first reported in 2006 [109]. Current challenges in boronate affinity materials demands new materials that can significantly enhance the interaction between boronate groups and glycopeptides and thus greatly reduce the nonspecific binding of non-glycosylated proteins and require no incubation with glycopeptides. The relatively weak boronate ester bond is also a challenge in boronate affinity materials. Formation of ester bonds between *cis*-diol and boronic acid occurs at solution pH above the pKa value of boronic acid group. Therefore, if the boronic acid moiety has lower pKa value, the binding is under mild pH conditions, and so higher binding affinity is expected [110]. For this, novel boronic acid ligands are explored, such as

those phenylboronic acids with electron-withdrawing groups, benzoboroxoles, heterocyclic boronic acids, etc. However, in addition to the specific *cis*-diol interaction, several secondary interactions, such as electrostatic, hydrophobic, and hydrogen-bonding interactions, are observed on boronic acid affinity materials. Among these, hydrophobic interaction could mostly influence the selectivity of boronic acid affinity. The most used aromatic boronic acid ligands show this hydrophobic interaction along with aromatic π interactions. To counteract the hydrophobic nature, hydrophilic boronic acid ligands are being explored. With the presented challenges and opportunities in boronic acid affinity binding, there are two main goals that are being sought in developing boronic acid affinity materials—maximum selectivity and highest binding capacity. High binding capacity of a material means high density of functional groups on its surface.

The porogen used in preparation of polymer monolithic materials not only solvates all agents in polymerization but may also affect the development of the porous structure. The use of polymer porogens has been suggested as a strategy to prepare macroporous monoliths. The use of porogenic poor solvents in preparation of ordinary polymer monolith leads to fast phase separation of the growing polymer chains and porogenic solvent. This results into coarsening of the monolithic structure and to heterogeneous microporous structures composed of tiny micron-size globular particles [1]. Polyethylene glycol (PEG) 10,000 MW has been used as macromolecular porogen in preparation of boronate affinity monolith in capillaries [111]. A linear macromolecule, polystyrene (PS), was also used as porogenic solvent to prepare a poly(VPBA-co-EDMA) monolithic column [78]. In polymerization, 4-vinylphenylboronic acid (4-VPBA) is a functional monomer, ethylene glycol dimethacrylate (EDMA) a crosslinker monomer, and a mixture of PS solution in tetrahydrofuran and toluene is a porogen. It was found that the selectivity of the boronate-affinity monolith can be tuned by varying the molecular weight and concentration of PS dissolved in the porogen mixture. The best polymerization condition was at monomer-to-crosslinker ratio (M/C) of 7:3 and PS concentration of 40 mg/mL. The developed poly(VPBA-co-EDMA) monolithic column was used for the separation of *cis*-diol flavonoid glycosides isomers—isoquercitrin (ISO) and hyperoside (HYP).

Non-specific adsorption of proteins could be observed when using boronate affinity columns with hydrophobic aromatic boronate compounds as affinity ligands. Therefore, hydrophilic macromonomer oligo (ethylene glycol) methyl ether methacrylate (OEG) was mixed with 3-(acrylamido)phenylboronic acid (AAPBA) as functional monomer to create a boronate affinity monolithic capillary with improved affinity [78]. The monolith was prepared using in situ polymerization using ethylene glycol dimethacrylate (EDMA) as crosslinking monomer and n-propanol and 1,4-butanediol as binary porogens. The resulting poly(AAPBA-co-OEG-co-EDMA) monolith was uniform in structure and had good column permeability. Improved recovery of HRP (97.51%) and ovalbumin (OVA) (93.97%) in polymer monolith microextraction (PMME) using the prepared OEG boronate monolith was observed. i.e., with an increase of 30% as compared to OEG-free boronate monolith (Figure 3). Good recoveries were also obtained during extraction of HRP (96.10%) and OVA (92.24%) from egg white samples.

The hydrophilic monomer 4-vinylphenylboronic acid (VBPA) was also used as the organic monomer to reduce the non-specific interactions due to the hydrophobic nature of the boronate-affinity monolith [79]. The preparation of hybrid monolith was done via a simple and convenient "one-pot" approach using divinylbenzene (DVB) as crosslinker along with VBPA. The binding pH of the prepared hybrid monolithic column was as low as pH 7.5. The prepared hybrid monolith combined hydrophilicity, cation exchange, and boronate affinity. The retention behavior of the prepared hybrid monolithic column was evaluated using small neutral molecules, aromatic amines, and *cis*-diol compounds.

One strategy to attain high adsorption capacity is via surface modification of the monolith with nanoparticles (NPs). The effective surface area and availability of interaction sites may be improved by incorporating nanoparticles into polymer monoliths. Graphene oxide (GO) exhibits high affinity and adsorption capacity for hydrophobic compounds and

carbon-based ring structures. A poly(vinylphenylboronic acid–ethylene glycol dimethacrylate) (VPBA-EDGMA-GO) monolithic material with GO incorporated was synthesized inside a poly(etherether ketone) (PEEK) tube as a sorbent for in-tube SPME [80]. In-tube SPME is a sample preparation technique being coupled to chromatography techniques for the analysis of complex biological samples. PEEK tube is a choice in column preparation due to its higher strength and pressure tolerance as compared to fused silica capillaries. An online SPME-HPLC system was prepared that coupled a boronic acid affinity monolith in a PEEK tube with a HPLC system. The addition of GO greatly improved the extraction efficiency of the boronate affinity monolith for HRP, and a limit of detection of 0.01 μg mL^{-1} was found using UV detection.

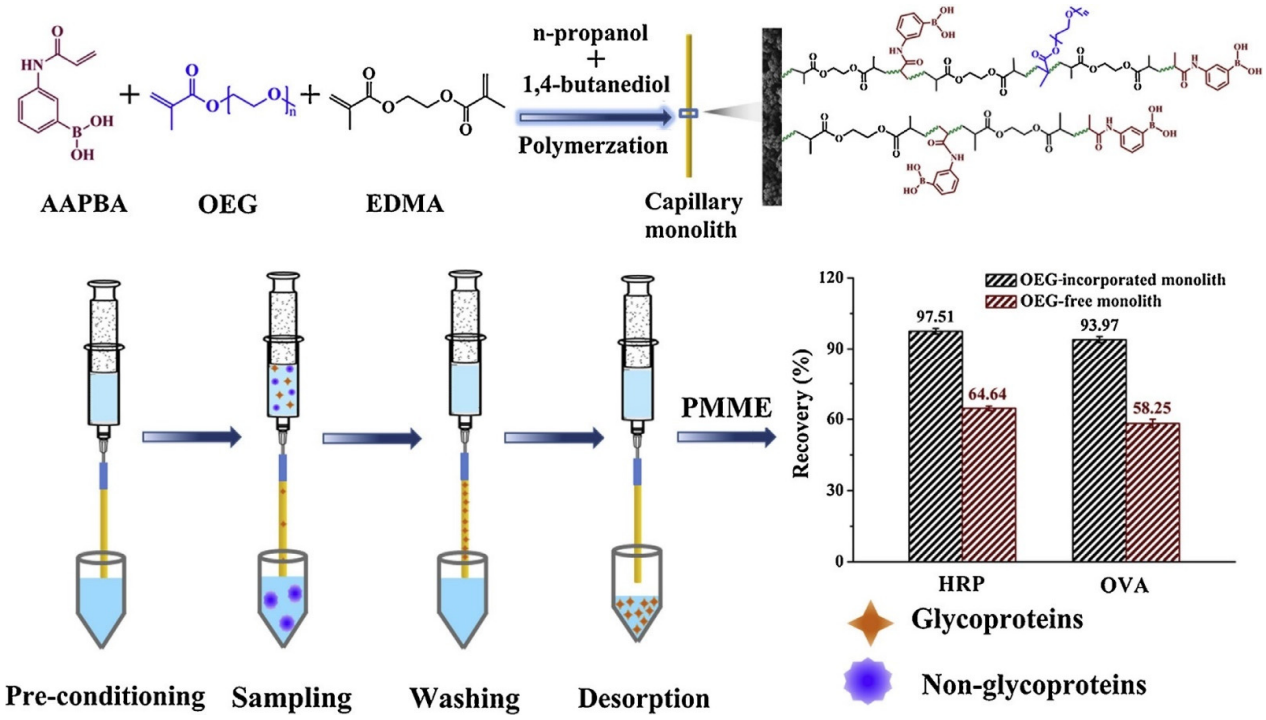

Figure 3. Schematic of synthesis and PMME analysis of HRP and OVA using poly(AAPBA-co-OEG-co-EDMA) monolithic column. Reprinted with permission from reference [78].

FSNPs was incorporated into hybrid monolithic column functionalized with 3-aminophenylboronic acid (3-APBA) resulting in poly(HPMA-Cl-MFSNP-EDMA) monolithic columns and were used in nano-liquid chromatography for separating glycoproteins [81]. Fumed silica nanoparticles (FSNPs) contain hydroxyl groups on their surface that facilitates easy chemical functionalization. The column was prepared in a 100-μm capillary by an in situ copolymerization of methacryloyl-fumed silica nanoparticle (MFSNP), 3-chloro-2-hydroxypropyl methacrylate (HPMA-Cl), and ethylene dimethacrylate (EDMA) in a binary porogenic solvent composed of cyclohexanol and dodecanol. Results showed that MFSNP content greatly affects the separation performance of the column. The chromatographic performance of the monolith was evaluated by the separation of alkylbenzene derivatives, proteins, and glycoprotein. The column efficiencies of 15,600–25,000 plates m^{-1} at the velocity of 1.2 mm s^{-1} was reached for alkylbenzenes in nanoliquid chromatography.

Conventional methods to lower the pKa of boronate affinity ligands involve using boronic acid ligands modified with electron-withdrawing groups, introduction of intramolecular tetracoordinated B-N or B-O bonds in Wulff-type boronic acid, and some other heterocyclic boronic acids [112]. 3,5-difluoro-4-formyl-phenylboronic acid (pKa = 6.5) was used as functional boronate affinity ligand to prepare an affinity silica hybrid monolith [82]. This functional ligand was covalently attached to the initially prepared amino-functionalized hybrid monolith. The resulting boronate affinity silica hybrid monolith was

able to specifically capture nucleosides under physiological conditions (at pH 6.5). The binding capacity for adenosine was found to be 693 μg mL^{-1} with good repeatability.

3-aminopropyltriethoxysilane-methacrylic acid (APTES-MAA) is an organic-inorganic hybrid monomer that has an acrylamino group that can coordinate with boronate ligands. Polyhedral oligomeric silsesquioxanes (POSS) is another organic-inorganic hybrid monomer that can be readily incorporated into polymer materials by copolymerization to improve mechanical and solvent-resistant properties. A boronate affinity monolith was prepared using these two organic-inorganic hybrid monomers (APTES-MAA and POSS) via one-step using 3-(acrylamido)phenylboronic acid (AAPBA) as boronate affinity ligand, ethylene glycol dimethacrylate as crosslinker, and iso-propanol and octanol as binary porogens [83]. The synergistic effect of APTES-MAA and POSS resulted in a boronate affinity monolith that developed good affinity and selectivity for glycoproteins (OVA, transferrin (Trf), HRP), good solvent resistance and pH stability, greater rigidity, and binds to glycoproteins at wide range of pH (5–8). A 1.7-fold greater glycoprotein recovery in polymer monolith microextraction (PMME) was observed using the prepared APTES-MAA/POSS-boronate affinity monolith as compared to an APTES-MAA/POSS-free monolith.

Another organic-inorganic hybrid is the polyhedral oligomeric silsesquioxane-methacryloyl histidine (POSS-MAH) monolithic column synthesized via free radical polymerization reaction [84]. Boronate affinity monolithic column was obtained when methacrylate (MA) of POSS was incorporated into the polymer chain by polymerization with amino acid based MAH monomer, which served as the electron donor to phenylboronic acid (PBA). The high density of the amino acid based MAH (NH_2 and imidazole) groups provides a large number of binding sites for boronate groups. Due to the high surface area obtained using the POSS monomer, a 6-fold to 7-fold higher in adsorption capacity (13.17 mg/g polymer) of (POSS-MAH-PBA) was achieved. The (POSS-MAH-PBA) boronate affinity monolithic column was 4.25 times more selective for adenosine than (POSS-MAH) monolithic column. The (POSS-MAH-PBA) boronate affinity monolithic column obtained an enrichment factor of 48.9-fold higher than that of (POSS-MAH) monolithic column. Recovery using (POSS-MAH-PBA) boronate affinity monolithic column was more than 90% for adenosine.

Molecularly imprinting polymers (MIPs) have also been used to create recognition sites on boronate affinity-based monoliths. MIPs are artificial recognition materials that offer excellent recognition ability with high stability at low cost and with easy preparation. A boronate affinity-based surface molecularly imprinted monolith (BA-SMIM) has been prepared in one study via simple two-step atom transfer radical polymerization (ATRP) strategy [85]. The preparation combined use of pseudo-template, surface imprinting, and covalent imprinting. Using pseudo-template and surface imprinting are strategies to avoid template leakage, which is a frequent problem for MIPs. The resulting monolith contains more homogeneous imprinted sites with no template leakage. The recognition capacity for *cis*-diols was due to the imprinting effect of BA-SNIM. Two *cis*-diol-containing compounds were retained longer than one *cis*-diol-containing compound because they interact synergistically to two imprinting cavities on BA-SMIM. The imprinted cavity of BA-SMIM was found subject to a nanoscale confinement effect that reduced the capturing pH for one *cis*-diol-containing compounds (from 8.6 to 7.8) and for two *cis*-diol-containing compounds (from 7.4 to 7.0). The nanoscale confinement effect gives the ligand molecule more affinity towards target molecules because its properties dramatically changed when confined in a limited space (complementary cavity for MIPs).

In-line coupling of capillary columns is an effective strategy for achieving miniaturized and automated separation methods for analysis of complex mixtures at trace levels. Miniaturization of chromatographic separations offers several advantages, such as low sample requirement, short analysis time, and use of smaller amounts of reagents. Miniaturized boronate affinity monolithic column (μBAMC) was prepared and adapted to in-line coupling with capillary zone electrophoresis [86]. This allowed a fully automated system that includes preconcentration/purification, separation and detection for the analysis of *cis*-diol

neurotransmitters in complex matrices. μBAMC was created by fabricating a short segment (1–3 cm) of silica-based monolith synthesized in situ at the inlet of a 75-μm i.d. fused silica capillary, followed by functionalization with 3-(acrylamido)phenylboronic acid (AAPBA) via free radical photopolymerization (10 min irradiation time). 1,2-dihydroxybenzene (catechol) was preconcentrated in a pH 8.5 phosphate buffer/MeOH (80/20 v/v) mixture. The developed in-line coupling (Figure 4) was successfully used in fully automated analysis of catecholamines neurotransmitters in urine samples. This developed method required low sample volume (less than 2 μL) and improved limits of detection (LOD) for dopamine, adrenaline, and noradrenaline compared to other CE methods estimated at 9.0, 9.5, and 4.8 ng mL^{-1}, respectively.

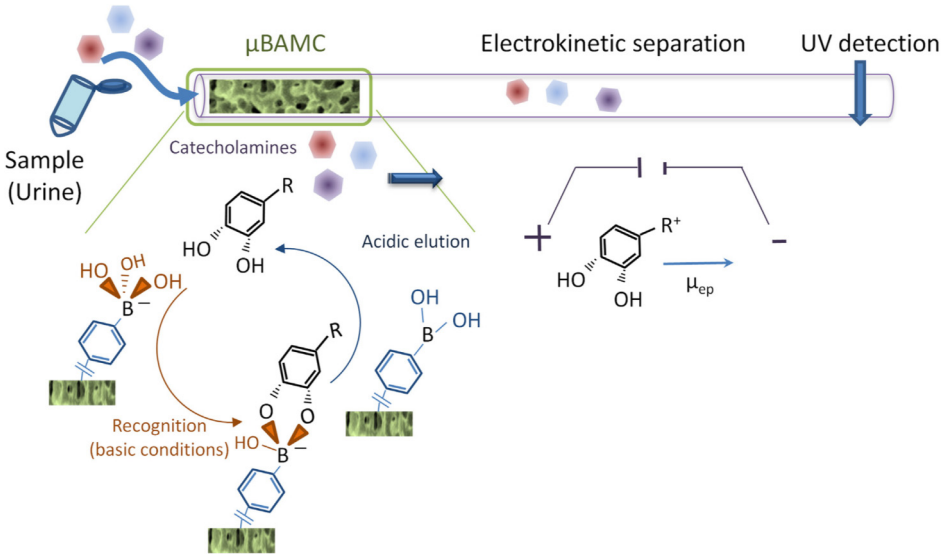

Figure 4. Schematic illustration of the different steps of *cis*-diols compounds analysis: preconcentration and purification on the integrated μBAMC unit, acidic elution and capillary zone electrophoresis separation, and UV detection. Reprinted with permission from reference [86].

Another study using an AAPBA-functionalized silica monolith in an in-line coupling of a boronate affinity purification/preconcentration step connected to a reversed-phase separation was reported [87]. The boronate affinity capillary column was prepared via thiol-ene photoclick reaction, wherein an increase in the number of phenylboronate sites by a factor of four was achieved compared to a photopolymerization reaction. This in-line coupling set-up consisted of a 1-cm length μBAMC unit connected to a 7-cm length reversed-phase monolithic column. This set-up in nano-LC experiments allowed control of injection volume and use of a large diversity of mobile phases due to the absence of pump and injection valve. This in-line coupling was successfully applied to the preconcentration and separation of four nucleosides (uridine, cytidine, adenosine, guanosine).

A macroporous polymer structure decorated with boronic acid was prepared using a crosslinked polyvinyl alcohol (MP-PVA) matrix [88]. Macroporous polymer structures are formed by phase separation, and the two-phase structure is fixed by additional cross-links. MP-VPA matrix was prepared by two crosslinking steps, first by self-crosslinking of the oxidized PVA then followed by glutaraldehyde cross-linking. This preparation was done in aqueous media without the use of an outer porogen. The resulting MP-PVA matrix was non-swellable and highly crosslinked with a heterogeneous structure consisting of globule-like structure (macropores, 100–300-nm dia.) and large pores several microns in size. The matrix had high pore volume and pore surfaces presenting reactive aldehyde groups. These aldehyde groups were utilized in immobilization of aminophenylboronic acid to create the hydrophilic boronate affinity monoliths. The prepared monoliths were then used to quantify glycated and non-glycated hemoglobin in human blood from diabetic patients.

An interface-free 2D monolithic material was prepared by coupling a thiol graphene (TG) doped poly(ionic liquid (ViOcIm$^+$Cl$^-$)) boronate affinity monolith to a poly (guanidinium ionic liquid) monolith [89]. The boronate-functionalized graphene monolith served as the preconcentration segment, while the poly(guanidinium ionic liquid) monolith served as the separation part. These coupled monoliths were used in multidimensional separations that allowed for analysis of glycoproteins in complex mixtures. Multidimensional systems in separation are usually a combination of multiple separation modes or multiple types of interfaces. An interface-free multidimensional separation system allows online sample concentration and separation while avoiding dead volume along the coupled materials. Uniform pore structure well attached to the inner wall of the capillary was observed, and there was no blocking at the border of the two segments. The preconcentration monolithic material exhibited good retention for five glycoproteins—HRP, OVA, AFP, TF, and RNase A. The binding capacity for these glycoproteins was determined at pH ranging from 5.0 to 9.0, specifically up to 10.16 and 10.47 mg/g at pH 9.0 for disease markers TF and AFP, respectively. High separation efficiency was attained using CEC in isolating glycoproteins from other non-glycoproteins. This could be due to the strong biological activity and unique molecular recognition ability of guanidinium. The resolution of the separation of AFP from HRP was improved when the monolith length was increased from 10 cm to 18 cm (Figure 5).

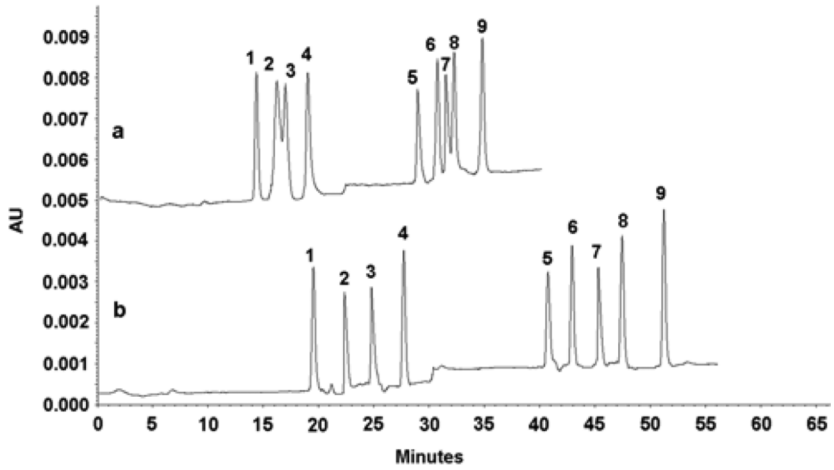

Figure 5. Effect of the length of separation monolith on the separation performance: (**a**) 10 cm and (**b**) 18 cm. Analytes: 1, BSA; 2, Mb; 3, Cyt c; 4, Lyz; 5, OVA; 6, TF; 7, AFP; 8, HRP; and 9, RNase A. Reprinted with permission from reference [89].

4.4. New Strategies on Separation and Enrichment of Glycans That Use Monolithic Materials

β-Cyclodextrin vesicles (CDVs) were grafted into mesoporous poly(glycidyl methacrylate-pentaerythritol triacrylate) (poly-(GMA−PETA)) monoliths via click reaction to prepare a pH-responsive monolith developed for capture and release of myoglobin (Myo) [90]. The CDVs were pre-decorated with SH-octapeptide (SH-CDV) to increase the encapsulation and improve the release rates of Myo prior to immobilization onto the monolith. CDVs are amphiphilic macrocycle vesicles formed by self-assembly. Due to their hollow and enclosed structures, they are capable of being responsive to electro-, thermo-, UV-, pH-, or chemo-stimuli [91]. CDV present an external surface of β-CD that can simultaneously capture multiple ligands and therefore can enhance the binding affinity and selectivity. The prepared SH-CDVs were spherical at pH 7.4 but transformed into fibers at pH 5.0. Due to this pH-sensitivity, this was used to controllably enrich myoglobin (Myo) glycopeptides from a mixture (1:10,000) of glycopeptides and non-glycoprotein BSA trypsin digests. Using this monolith combined with MALDI-MS detection, a detection limit of 0.1 fmol was obtained, and 15 glycopeptides from Myo digest were captured. Moreover, 166 intact glycopeptides from 130 glycoproteins in human blood samples were able to be

identified. Cyclodextrins are well-known as host molecules that contain a hydrophobic cavity and hydrophilic upper and lower rims; due to the molecular structure of CDs, they are very versatile in hosting various guest molecules [113].

Cyclodextrin molecular tube (CDMT) was functionalized with glutamate, immobilized into a solid monolithic support by a host−guest self-assembly synthetic strategy and used as an enrichment platform for specific capture of glycopeptides [92]. CDMT is a hollow, tubular polymer formed by threaded CDs and used as a building block in self-assembled structures [114]. CDMT has a large and hydrophilic external area and is a stable structure. Functionalization with glutamate (gluCDMT) resulted in an even larger hydrophilic surface that was still stable. The hydrophilic sites were still available after immobilization in monolith. A high binding capacity (~50 mg g^{-1}) was observed on this gluCDMT-based monolith, and it was able to capture glycopeptides (23 HRP glycopeptides and 28 IgG glycopeptides). The glycopeptides were captured from HRP/BSA mixture (1:10,000), showing the good selectivity of this gluCDMT-based monolith. Analysis of glycopeptides in acute myelogenous leukemia cell lysate and human serum samples was also done using this developed platform.

A new method in separation of glycans was reported that uses fullerenes. C_{60}- and C_{70}-fullerene bonded columns (C_{60} and C_{70} columns) were prepared using a silica monolithic capillary and perfluorophenyl azide as a thermo-reactive agent [93]. Fullerenes have high electron density on their aromatic rings and therefore provide specific π interactions to glycans, such as CH- or OH-π interactions, along with dipole interactions. As the number of sugar units increases, the interactions between sugar and fullerene increase. The developed monolithic columns were used to separate 2-aminobenzamide-labeled glucose homopolymers (Glcs) as from non-labeled glucose homopolymers by LC under aqueous conditions. Note that in HILIC, the use of aqueous solvents with higher fraction of water as mobile phase causes interference on interactions, as a hydration sphere provides specific interaction with polar compounds, such as sugar. The retention rates of disaccharides, such as maltose, trehalose, and sucrose, were determined using C60 column. It was found that maltose was retained better than other disaccharides due to its higher dipole moment.

A customized chromatographic monolithic column was prepared for fast and simple immunoaffinity purification of fibrinogen (FIB) from human blood samples using monoclonal anti-human fibrinogen antibodies [94]. FIB is a secretory glycoprotein synthesized by hepatocytes that is important for blood clotting. Monoclonal anti-human fibrinogen antibodies were covalently crosslinked to the protein A-based monolith using the homobifunctional reagents dimethyl pimelimidate (DMPI) and dimethyl suberimidate (DMSI), reacting with primary amino groups on both proteins to form amidine bonds. The developed monolith in column was used in FIB immunoaffinity isolation using HPLC followed by *N*-glycosylation analysis via ultra-performance liquid chromatography.

An amorphous titania monolith modified with boric acid was used in solid-phase extraction of glycoproteins (ribonuclease B, HRP, OVA) [95]. It was already observed that incorporation of titania can produce a more hydrophilic boronate-functionalized hybrid (silica/titania) monolithic column [115]. The non-aqueous sol-gel route was used to prepare borated titania monoliths for the capture of *cis*-diol-containing molecules [116]. Inorganic boric acid was expected to enhance the hydrophilicity and therefore also the selectivity of the material towards glycoproteins. Glycoproteins were captured in slightly basic solutions and released in acidic solutions, then detected by MALDI-TOF mass spectrometry. The observed binding capacities were 9.3, 26.0, and 53.0 mg g^{-1} for ribonuclease B, HRP, and OVA, respectively. The potential of these monoliths for analysis of real samples was demonstrated by selective enrichment of OVA from egg white sample.

Polymer monolith microextraction (PMME) uses a polymer monolith that has porous structure, rapid mass transfer, good biocompatibility, and is also easy to prepare in capillary tubes [117]. PMME has usually limited functional groups that affect its extraction efficiency when used in bioseparations, such as of glycans; therefore, studies are focused on modifying polymer monoliths with functional groups. Cobalt phthalocyanine tetracarboxylic acid

(CoPcTc) was grafted into poly(GMA-EDMA) monolith using condensation acylation of carboxyl groups with amine groups to fabricate a poly(GMA-EDMA-CoPcTc) monolith for PMME [96]. The polymer monolithic material was coupled with MALDI–TOF MS and used for glycopeptide enrichment. The coordination interaction between cobalt and glycopeptides also includes hydrogen bonds between isoindole subunits of phthalocyanine. Twenty-eight and seventeen glycopeptides from IgG and HRP were identified, respectively. High enrichment selectivity of IgG glycopeptides was also observed even in the presence of a large interference (BSA) ratio (50:1, m/m) (Figure 6). It was also applicable for trace analysis, as the detection limit obtained was 6.7 fmol. Enrichment of N-linked glycans from human serum samples was achieved using this monolith.

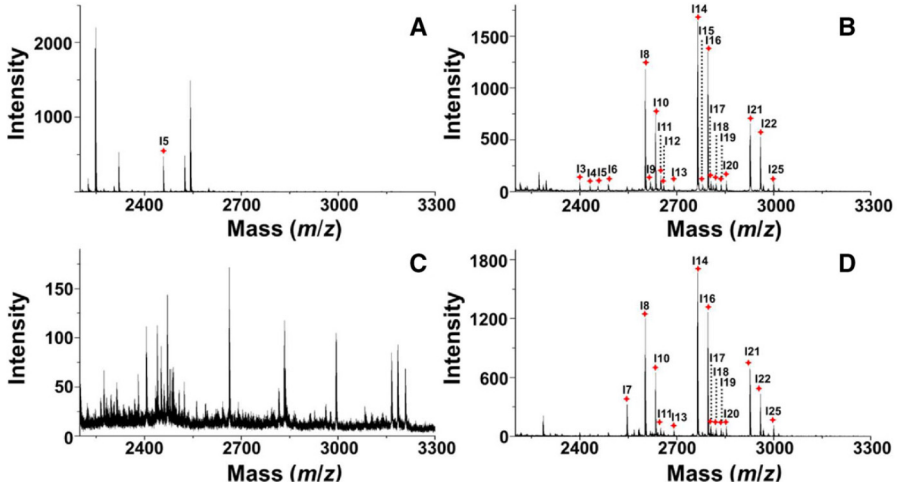

Figure 6. MALDI–TOF mass spectra of the mixture of BSA and IgG digests. (**A**) Direct analysis at a mass ratio of 10:1 (**B**) after enrichment by poly(GMAEDMA-CoPcTc) monolith at a mass ratio of 10:1, (**C**) direct analysis at amass ratio of 50:1, and (**D**) after enrichment by poly(GMA-EDMA-CoPcTc) monolith at a mass ratio of 50:1. Reprinted with permission from reference [96].

Another PMME monolith was prepared using poly(glycidyl methacrylate-ethyleneglycol dimethacrylate) functionalized with copper tetra(N-carbonylacrylic) aminephthalocyanine (CuMPc) and iminodiacetic acid (IDA) and used in selective enrichment of HRP glycopeptides [97]. CuMPc is a water-soluble derivative of metallophthalocyanines (MPcs), whose indol-like structures could interact with glycans through hydrogen bonds with the planar polar nitrogen groups of the phthalocyanine (Pc) ring [118]. IDA was incorporated due to its zwitterionic hydrophilic properties, and zwitterion-based monoliths have already showed potential in selective isolation and enrichment of glycopeptides [98]. This poly(GMA-EDMA-CuMPc-IDA) monolith was used in PMME coupled with MALDI-TOF-MS to identify glycopeptides in a complex biological sample. A total of 24 glycopeptides were identified with a detection limit of 5 fmol from tryptic digest of immunoglobulin G. A total of 16 glycopeptides were still identified even when IgG was in a mixture with bovine serum albumin (1:100 m/m). The monolith was also used in selective enrichment of glycopeptides from human digests. Another poly(GMA-EDMA-CuMPc-IDA) monolith was used in PMME coupled with MALDI-TOF-MS to enrich and analyze HRP tryptic digests [98]. The tryptic digests of HRP were enriched owing to H-bonding and hydrophilic interactions. A total of 20 glycopeptides were captured and identified with a detection limit as low as 0.5 fmol μL^{-1}. High selectivity was also observed when 14 glycopeptides were identified even from a mixture with BSA and HRP digests (200:1, m/m). The developed poly(GMA-EDMA-CuMPc-IDA) monolith exhibited good stability and reproducibility.

4.5. Additional Strategies for Separation and Enrichment of Glycans and Other Cis-Diol Molecules

There are recent works that prepared materials for separation and enrichment of glycans and other *cis*-diol containing molecules without using a monolith (Table 2). The strategies on these methods are worth adding on the list, as they could be adapted in developing new monolithic materials for separation and enrichment of glycoproteins.

Table 2. Additional strategies in separation and enrichment of glycans and other *cis*-diol molecules.

Strategies	Application to Separations	Ref/Year
Grafting of boronic acid ligands on silica by surface-initiated atom transfer radical polymerization (SI-ATRP) to create silica-pAAPBA-PBA adsorbent	Excellent selectivity and a higher binding capacity for catechol (513.6 mmol g^{-1}) and for fructose (736.8 mmol g^{-1})	[119] 2015
Grafting benzoboroxole to dendrimer beads to create synergistic benzoboroxole–glycan interactions with multiple monosaccharides in which one sugar bears several OH groups	Increased enrichment efficiency for glycopeptides; identified over 1000 N-glycosylation sites in yeast, 4195 sites on 1608 N-glycoproteins in mouse brain tissues, and 4691 sites on 1906 N-glycoproteins in human cells	[120] 2018
Phenylboronic acid (PBA) introduced to SiO_2 microspheres by a thiol-ene click chemistry method	High selectivity for both neutral and acidic glycopeptides due to synergistic effects of affinity interaction and hydrophilic interactions	[121] 2017
Grafting phenylboronic acid onto the surface of MOF UiO-66-NH_2 nanoparticles through amidation reaction to create dual-functionalized magnetic MOFs nanoparticles with abundant amino groups and grafted phenylboronic acid	High binding capacities toward glycoproteins (OVA—327.28 mg/g, Trf—241.17 mg/g, HRP—530.79 mg/g) was observed under physiological state (pH 7.4) due to both hydrophilicity and boronic acid affinity	[122] 2018
Double recognition due to boronic acid-functionalized graphene oxide and molecularly imprinted spatial matched cavities for OVA	High binding capacity (278 mg/g) and fast adsorption/elution rate (within 40 min) for OVA	[123] 2017
Immobilization of boronic acid ligands on magnetic Fe_3O_4 nanoparticles using distillation precipitation polymerization (DPP) to create core-shell structured Fe_3O_4@P(AAPBA) (poly 3-acrylaminophenylboronic acid) and Fe_3O_4@P(AAPBA-comonomer) hydrophilic magnetic nanoparticles	Enhanced binding strength and selectivity towards glycoproteins due to plentiful boronic acid and its synergistic effect with hydrophilic monomers	[124] 2015
Boronic-acid-functionalized magnetic graphene (graphene@phenolic-formaldehyde (magG@PF@APB)) resin multilayer composites	Large specific surface area, strong magnetic responsiveness, biocompatible, and enhanced affinity; low detection limit (1 fmol) and good selectivity (1:100)	[125] 2015
Using magnetite colloid nanocrystal clusters (MCNCs) as the "core" and the phenylboronic acid-modified covalent organic frameworks (COFs) as the "shell" (MCNCs@COF@PBA)	Outstanding selectivity (HRP:BSA = 1:600), good sensitivity (100 amol), high enrichment recovery (~93% ± 3%), and rapid magnetic separation (~1 min)	[126] 2019
Grafting allose units into a polyacrylamide chain to create a saccharide-based sialylated glycopeptides (SGs) receptor	High-performance enrichment capacity towards SGs; identified 180 SGSs that are much higher than those identified by SA-binding lectins, such as WGA (18 SGSs) and SNA (22 SGSs)	[127] 2016
Use of oligopeptides (with optimal dipeptide sequences) screened out using a hydropathy-index-based strategy	Excellent glycopeptide enrichment, i.e., selectivity up to ~70% for real biosamples; can discriminate isomeric glycosidic linkages	[128] 2016
Grafting random copolymer brushes on silica nanoparticles to create Si@pNIPAm-b-pBA nanohybrid material	High binding capacities for OVA (98.0 mg g^{-1}) and HRP (26.8 mg g^{-1}) were achieved with a low steric hindrance	[129] 2017
Creating boronic acid brushes on the microsphere surfaces resulting to APBA@PGMA/EDMA microspheres	Excellent adsorption selectivity and high extraction efficiency for glycoproteins	[130] 2017
Grafting polymer brush using Poly(3-acrylamidophenylboronic acid) (PAAPBA) via surface-initiated atom transfer radical polymerization (SI-ATRP)	Successfully used in enrichment of catecholamines from real urine samples	[131] 2018
Use of attapulgite (a fibrous aluminum-magnesium silicate) grafted with a 1,3,5-triazine-containing binary boronic acid	Able to bind to *cis*-diols at lower pH (5.0); high adsorption capacity (19.5 ± 1.1 mg·g^{-1}) for adenosine; high selectivity for *cis*-diols (1:1000)	[132] 2016
Use of Ugi ligand, A21C11I8, that is comprised of benzoboroxole (cylic boronic acid derivative) on aldehyde-functionalized Sepharose™	Able to purify Gox from spiked *E. coli* supernatants at neural pH with 98% purity; able to resolve sialylated and neutral glycoforms	[133] 2016

Table 2. *Cont.*

Strategies	Application to Separations	Ref/Year
Coating the surface of thin-film stainless steel blades with boronate functionalized particles of phenylboronic acid (PBA) and 3-aminophenyl-boronic acid (3-aPBA) to create affinity solid-phase microextraction (BA-SPME)	Selectively extract and enrich glycoproteins (asialofetuin and lactoferrin); extraction and elution process can be easily controlled by adjusting the pH	[134] 2015
Use of N-succinimidyloxycarbonylmethyltris(2,4,6-trimethoxyphenyl)phosphonium bromide (TMPP-Ac-OSu) to label N-glycans after rapid deglycosylation	Over a 50-fold enhancement in the sensitivity for neutral glycans from RNase B as compared to their underivatized counterparts	[135] 2016
Use of readily available Protein-A column as mini affinity chromatography to IgG antibodies	N-glycans from monoclonal antibodies (mAbs) were isolated directly from cell culture supernatant in a method with high yield and non-invasive	[136] 2015
N-octyl-modified monodispersed dendritic mesoporous silica nanospheres (DMSNs) with small diameter (~170 nm), appropriate pore size (5.6 nm), and packed into capillaries (12-cm long)	Increase in the permeability of packed capillaries with ultrahigh efficiency up to 3,500,000 plates/m as evaluated in CEC mode; applied to glycan profiling of cancerous and normal cells	[137] 2021

Surface-initiated atom transfer radical polymerization (SI-ATRP) was used to control polymer chain length and create a high density of accessible boronic acid groups on the surface of a high-capacity boronate affinity adsorbent using 3-acrylamidophenylboronic acid (AAPBA) as the monomer [119]. In ATRP, simple organic halide groups, such as chlorines, are used as initiators during the polymerization reaction. These chlorine atoms were subjected to chain-end functionalization technology via "click chemistry" to attach more boronic acid molecules, resulting in silica-pAAPBA-PBA. The prepared material showed a high selectivity and a higher binding capacity for catechol (513.6 mmol g^{-1}) and for fructose (736.8 mmol g^{-1}). The silica-pAAPBA-PBA was also used to extract three *cis*-diol drugs (epinephrine, isoprenaline, and caffeic acid isopropyl ester) from plasma using dispersed solid-phase extraction (dSPE). The eluates analyzed by HPLC-UV showed only trace *cis*-diol targets, while non-*cis*-diols were nearly excluded, proving the specific selectivity of silica-pAAPBA-PBA material.

Using dendrimer-conjugated benzoboroxole is one proposed strategy to enhance the interactions between boronic acid ligand and glycopeptides and so to improve enrichment efficiency [120]. Benzoboroxole conjugated to a dendrimer resulted in synergistic benzoboroxole–glycan interactions. The developed benzoboroxole-conjugated dendrimer beads had multiple sites and were thus well suited to the common feature of glycans, i.e., their containing multiple monosaccharides and one sugar bearing several hydroxyl groups. Results showed that the enrichment efficiency was increased even for glycopeptides only containing β-linked N-acetylglucosamine (OGlcNAc). Identification of 4195 N-glycosylation sites on 1608 N-glycoproteins found in mouse brain tissues, of over 1000 such sites in yeast, and of 4961 sites on 1906 glycoproteins from human cells was accomplished, including for many glycoproteins of low abundance.

Phenylboronic acid (PBA) was introduced onto SiO_2 microspheres by a thiol-ene click chemistry method (click PBA) [121]. This developed boronate affinity material was used to separate *cis*-diol-containing molecules and enrich glycopeptides in HILIC mode. Due to synergistic effects of affinity interaction and hydrophilic interactions, click PBA exhibited high selectivity for both neutral and acidic glycopeptides. These glycopeptides were separated from 100 molar fold excess of bovine serum albumin digests. A total of 101 unique glycosylation sites from 71 glycoproteins were identified in 1 μL human serum.

A core-shell nanoparticle structure with a Fe_3O_4 magnetic core surrounded by hydrophilic Mg-MOF-74, which has a 1D channel porous structure, was prepared and used for detection of 441 N-glycosylation sites of 418 glycoproteins from 125 glycoproteins in 1 μL of human serum [138]. Metal−organic frameworks (MOFs) are porous materials composed of metal ions or metal clusters coordinated to organic ligands. MOFs material has low density, high specific surface area, uniformly structured cavities, and good thermal stability [139]. Magnetic MOF nanocomposites with 1,4-phenylenebisboronic acid were prepared and used for selectively capturing and releasing glycoproteins via pH-stimulus-response [140]. The

performances of these MOFs were limited by lack of functional ligand or low content of functional ligands. Due to this limitation, dual-functionalized magnetic MOFs nanoparticles were synthesized by grafting phenylboronic acid onto the surface of UiO-66-NH_2 through amidation reaction [122]. This dual property was due to the abundant amino groups and grafted phenylboronic acid groups resulting in both hydrophilicity and boronic acid affinity (Figure 7). High binding capacities toward glycoproteins (OVA—327.28 mg g^{-1}, Trf—241.17 mg g^{-1}, HRP—530.79 mg g^{-1}) was observed under physiological state (pH 7.4). The nanoparticle still gave excellent enrichment performance even after using for the sixth time.

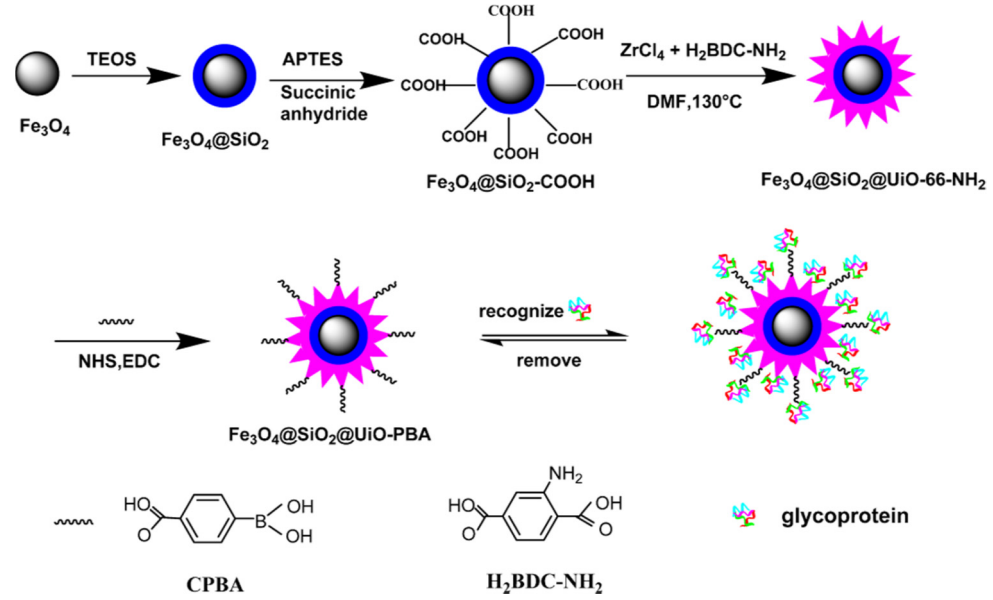

Figure 7. Schematic illustration of synthesis of a dual-functionalized magnetic MOFs nanoparticles by grafting phenylboronic acid onto the surface of UiO-66-NH_2 through amidation reaction. Reprinted with permission from reference [122].

A boronate affinity material with double recognition abilities was used as a highly efficient adsorbent for glycoproteins [123]. The double recognition ability is due to the combination of boronate affinity and molecularly imprinted, spatially matched cavities for OVA. First, boronic acid functionalized graphene oxide (GO-APBA) was prepared. Then, a template OVA was immobilized onto the surface of GO-APBA through boronate affinity. Sol-gel polymerization of organosilanes from aqueous solution was used to deposit an imprinting layer onto the surface of GO-APBA, and 3D cavities with double recognition abilities toward OVA were obtained after removing the template (GO-APBA/MIPs). The saturation binding capacity of GO-APBA reached 278 mg g^{-1} in 40 min, and a high binding capacity and rapid adsorption and elution for OVA interaction with GO-APBA/MIPs was found.

One way to improve boronic acid affinity material is by immobilization of boronic acid ligands on magnetic particles. A core-shell structured Fe_3O_4@P(AAPBA) (poly 3-acrylaminophenylboronic acid) and Fe_3O_4@P(AAPBA-comonomer) multifunctional magnetic nanoparticles were prepared using distillation precipitation polymerization (DPP) [124]. Hydrophilic Fe_3O_4@P(AAPBA-co-monomer) nanoparticles showed an enhanced binding strength and selectivity towards glycoproteins due to plentiful boronic acid groups and their synergistic effect with hydrophilic monomers. The hydrophilic Fe_3O_4@P(AAPBA-comonomer) microspheres were used to separate glycoproteins in physiological conditions (pH 7.4), and with the presence of an external magnetic field, captured glycoproteins were separated from the solution. The Fe_3O_4@P(AAPBA-co-monomer) nanoparticles were successfully used to selectively capture and identify the low-abundance glycopeptides from

the tryptic digest of horseradish peroxidase (HRP) and in the enrichment of glycoproteins from egg white samples.

A boronic-acid-functionalized magnetic graphene@phenolic-formaldehyde(mag G@PF@APB) resin multilayer composites was developed [125]. This affinity material exhibited a large specific surface area, a strong magnetic response, biocompatibility of the resin, and the enhanced affinity properties of boronic acid. The hydrophilic magG@PF@APB has well-defined 2D morphology and high loading amounts of boronic acid and so is suited for selective enrichment and separation of glycopeptides. In a mixture of glycopeptides and non-glycopeptides (1:100), a low detection limit (1 fmol) and good selectivity were achieved using the composites. The enrichment efficiency was retained even after using the composites for the third time. Successful enrichment of human serum of a low sample volume (1 µL) using these magG@PF@APB composites proved its potential in glycoproteome analysis of complex biological samples.

Formation of a core-shell structure of covalent organic framework modified by boronic acids around a magnetic core was used to create a new boronic acid affinity material (MCNCs@COF@PBA) [126]. The composite was fabricated using azide−alkyne cycloaddition ("click") reaction with magnetite colloid nanocrystal clusters (MCNCs) as the "core" and the phenylboronic acid-modified covalent organic frameworks (COFs) as the "shell." This composite material had magnetic properties, intrinsic mesoporous structure, large surface area, and an large number of affinity sites due to combined characteristics of Fe_3O_4 particles, COFs, and boronate affinity. It was then used as an adsorbent to efficiently and specifically capture N-linked glycopeptides from the digested glycoprotein and protein mixture while excluding proteins. This MCNCs@COF@PBA composite showed good performance in the enrichment of N-linked glycopeptides, i.e., outstanding selectivity (HRP:BSA = 1:600), good sensitivity (100 amol), high enrichment recovery (~93 ± 3%)m and rapid magnetic separation (~1 min). Furthermore, the developed MCNCs@COF@PBA-based MS method could be successfully applied to analyze glycopeptides found in exosomes secreted from HeLa cells.

Allose was utilized to create a saccharide-based sialylated glycopeptide (SGs) receptor used to enrich trace SGs in highly complex biological samples [127]. Allose is a sugar unit that specifically binds to sialylated glycopeptides (SGs), forming six hydrogen-bonds between allose and N-acetyl-neuraminic acid. Binding strength was tuned by varying the solution pH and polarity (acetonitrile/H_2O ratio). Allose units were grafted into a polyacrylamide chain using benzene ring linker to produce saccharide-responsive smart copolymer (SRSC). The use of SRSC showed high-performance enrichment capacity towards SGs even in a mixture with 500-fold bovine serum digests as interferences. Using this SRSC, 180 sialylated glycosylation sites (SGSs) were identified, much higher than the number identified by SA-binding lectins, such as WGA (18 SGSs) and SNA (22 SGSs).

Oligopeptides were utilized in designing a promising saccharide-binding platform for glycopeptide analysis [128]. Good affinity between oligopeptide and glycan is due to H-bonding interactions between amino acid residues of oligopeptides and hydroxyls of glycans. Specific recognition of various glycans is possible due to the flexible stereoconformations of oligopeptides that enables saccharide discrimination via variation in affinities. The binding can be optimized because the oligopeptide sequence is easy to design. A hydropathy-index-based strategy for sequence optimization was used to select three optimal dipeptide sequences from 54 types of dipeptides. The selected dipeptide-based homopolymers showed excellent performance in glycopeptide enrichment, i.e., selectivity up to ~70% for real biological samples and anti-interference capacity in a mixture with 1000-fold bovine serum albumin interference. The developed material could discriminate the slightly different glycan structures of glycopeptides, such as isomeric glycosidic linkages.

One drawback of boronic acids as glycoprotein adsorbents is the relative weakness of the boronate ester bond, and so it is difficult for a lone boronic acid to capture large biomolecules, such as glycoproteins. Use of synergistic multiple covalent bonds is one

strategy to respond to this challenge; in this way, multiple boronic acids will bond to glycans on glycoproteins for capture. Increasing the density of boronic acid groups on the supports by grafting polymer brushes presenting boronic acid groups onto the supports is one way of achieving this aim. Grafting polymer brushes could result in a soft boronate affinity material with both a high density of boronic acids with low steric hindrance. One study involved grafting random copolymer brushes onto silica nanoparticles for glycoprotein separation [141]. Steric hindrance was reduced using these long and flexible polymer brushes, which improved glycoprotein binding to boronic acids. Later, another study developed a method to immobilize a high density of boronic acid ligands on thermally responsive block copolymer brushes [129]. Block copolymer brush, pNIPAm-b-pGMA, was synthesized by a two-step SI-ATRP. After post-functionalization of the pGMA block with sodium azide, a high density of alkyne-tagged boronic acid was introduced to the polymer brushes via Cu(I)-catalyzed azide−alkyne cycloaddition (CuAAC) click reaction and obtained a new boronate affinity nanocomposite, Si@pNIPAm-b-pBA, with a potent glycan binding affinity (Figure 8). High binding capacities for OVA (98.0 mg g^{-1}) and HRP (26.8 mg g^{-1}) were achieved using the developed nanocomposite due to the high density of boronic acid ligands on the flexible polymer brushes that bind simultaneously via multivalent interactions with glycans. The binding conditions were in pH 9.0 buffer at 20 °C, which could be adjusted to tune the binding capacity of the nanocomposite.

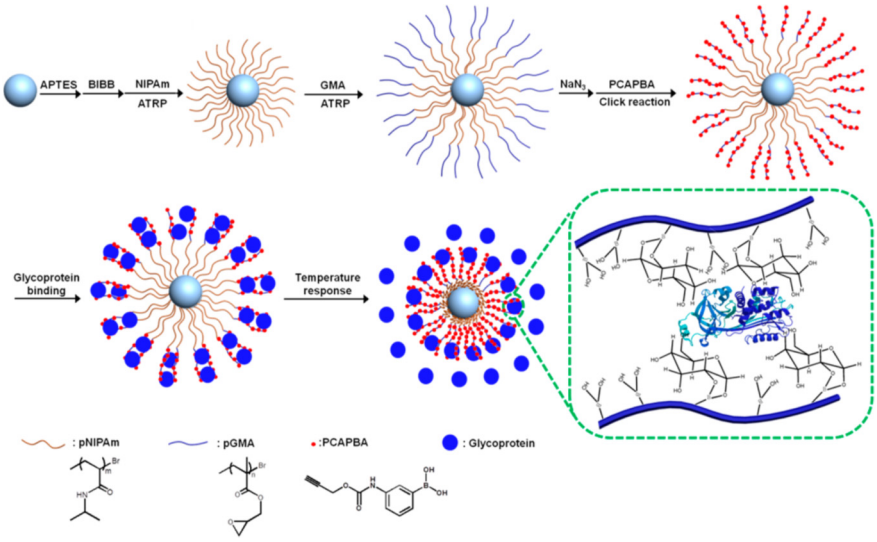

Figure 8. Preparation of Si@pNIPAm-b-pBA core-brush nanocomposite via the combination of SI-ATRP with CuAAC click reaction and the schematic of glycoprotein binding to the boronic acid-functionalized polymer brushes. Reprinted with permission from reference [129].

Well-defined boronic acid brushes were also prepared on the microspheres to create SPE matrices [130]. These microspheres were filled in a microcolumn. The microspheres were poly(glycidyl methacrylate-co-ethylene dimethacrylate) functionalized with 3-aminophenylboronic acid (APBA@PGMA/EDMA The APBA@PGMA/EDMA microspheres were found to have excellent adsorption selectivity and high extraction efficiency for glycoproteins. The APBA@PGMA/EDMA microcolumn could separate and enrich glycopeptides containing multiple N-linked and O-linked glycosylation sites from enzymatic hydrolysate derived from human serum.

Another study grafted polymer brushes from polydopamine-coated magnetic graphene oxide onto poly(3-acrylamidophenylboronic acid) (PAAPBA) via SI-ATRP [131]. The prepared boronate affinity material was readily dispersed in water and had a good adsorption capacity for *cis*-diols, such as adenosine, salbutamol, dopamine, and catechol. This BAM could selectively capture *cis*-diols from non-*cis*-diols in a mixture containing molecules without nitrogen atoms. Some non-*cis*-diols were retained but much fewer than the *cis*-diols

for the ones with nitrogen atoms. This BAM was successfully used in enrichment of catecholamines from urine samples.

Attapulgite, a fibrous aluminum-magnesium silicate was grafted with a 1,3,5-triazine-containing binary boronic acid (DBA) [132]. The binary boronic acid was prepared by reacting cyanuric chloride with 3-aminophenylboronic acid. The resulting functionalized attapulgite binds *cis*-diols at lower pH (5.0) due to the strong electron-deficient 1,3,5-triazine ring. It also showed high adsorption capacity (19.5 ± 1.1 mg g^{-1}) for adenosine due to the two binding sites and large specific surface area of DBA. It has good selectivity for *cis*-diols in the presence of a thousand-fold excess of interfering species. The prepared material was used in selective extraction of nucleosides from spiked human urine with recoveries found between 85.0 and 112.9%.

A Ugi ligand, A21C11I8, which was comprised of a benzoboroxole derivative 5-amino-2-hydroxymethylphenylboronic acid was synthesized on aldehyde-functionalized Sepharose^{TM} [133]. Benzoboroxole is a type of cyclic boronic acid derivative that is a very stable, water-soluble boronic acid that could be used to enrich glycoproteins under physiological conditions [142]. Immobilized A21C11I8 has preference for sugar alcohols and the furanose form of monosaccharides. This Ugi ligand was able to purify Gox from spiked *Escherichia coli* supernatants at neutral pH with 98% purity and maximum enzymatic activity retaining the biological activity of GOx. This developed adsorbent was able to resolve sialylated and neutral glycoforms.

Boronate-functionalized particles of phenylboronic acid (PBA) and 3-aminophenylboronic acid (3-aPBA) were used to coat the surface of thin-film stainless steel blades. These blades were then used as a new extraction phase based on boronate affinity solid-phase microextraction (BA-SPME) [134]. This prepared material selectively extracted and enriched glycoproteins (asialofetuin and lactoferrin) from a mixture with non-glycoproteins (BSA and myoglobin). The process of extraction and elution was easily controlled by adjusting the pH. The extraction method was further used in extracting glycoproteins from standard buffer, PBD, human plasma, and 10-fold diluted human blood.

MALDI-MS offers several advantages in glycan analysis, such as simple operation, short analysis time, and ability to be carried out in the presence of impurities [143]. However, low ionization efficiency of N-glycans in MALDI-MS is observed due to hydrophilic OH groups of N-glycans and also lack of basic sites of protonation. One conventional strategy is glycan labeling via reductive amination using chemical agents, such as 2-aminobenzamide (2-AB) or 2-aminobenzoic acid (2-AA) [144]. Carbamates or activated esters are also used to derivatize glycosylamines after release from Peptide-N-glycosidase F (PNGase F) deglycosylation. One challenge for this is the rapid hydrolysis of glycosylamines after overnight digestion such that they can no longer be derivatized. A new derivatization method was proposed that used N-succinimidyloxycarbonylmethyltris(2,4,6-trimethoxyphenyl)phosphonium bromide (TMPP-Ac-OSu) after rapid deglycosylation [135]. Labeling was done in a one-pot reaction that introduces a permanent charge at the reducing end of N-linked glycans. Here, glycosylamines were released after rapid (5 min) deglycosylations using an enzyme-friendly mild detergent N-dodecyl β-D-maltoside (DDM). Using this derivatization method, more than 50-fold enhancement in the sensitivity was achieved for neutral glycans from ribonuclease B (RNase B) as compared to their underivatized counterparts. In addition, 50 glycans were detected using only 25 nL human serum sample of sialoglycoproteins by using this derivatization method.

Readily available Protein-A columns were used in mini-affinity chromatography to first capture immunoglobulin G (IgG) antibodies. N-glycans from the monoclonal antibodies (mAbs) were isolated directly from cell culture supernatant in relatively rapid, highly reproducible, and inexpensive method [136]. In a single process, mAbs were captured, followed by PNGase F digestion to release their N-glycans. The method is high in yield and non-invasive and allowed quantification of both antibody and glycan concentrations in a single analysis. Collected antibodies were quantified using a UV-vis

spectrophotometer. The released N-glycans were quantified by 2-aminobenzamide (2-AB) labelling followed by HILIC-based HPLC analysis.

Novel n-octyl-modified monodispersed dendritic mesoporous silica nanospheres (DMSNs) were controllably synthesized and could be the smallest minimum particle size in liquid chromatography that exhibited ultra-high efficiency [137]. These nanomaterials have small diameter (~170 nm) and appropriate pore size (5.6 nm) and were packed into capillaries (12-cm long) using only regular HPLC pump. Their unique morphology was described as center-radial centrosymmetric mesopore channels. Their small particle size and highly uniform packing significantly improved the permeability of packed capillaries and exhibited ultra-high efficiency up to 3,500,000 plates m^{-1}, as evaluated in CEC mode. The developed CEC method was applied to profile the glycans of cancerous and normal cells. Due to the obtained high-efficiency N-glycan profiles, results revealed that cancerous cells exhibited characteristic N-glycans distinct from those on normal cells.

5. Immobilized Monolithic Enzyme Reactors (IMERs)

In sample preparation in glycoproteomics, glycoproteins undergo proteolysis during which they are cleaved into peptides and glycopeptides. Trypsin is the most commonly used enzyme in glycopeptide analysis and cleaves peptide bonds on the C-terminal side of arginine and lysine residues [145]. Complementary to proteolysis, glycosidase digestion to release glycans can be used for glycopeptide-based analysis. PNGase F is an example of a glycosidase that cleaves N-linked glycans between innermost GlcNAc and asparagines from N-linked glycoproteins [146]. The conventional method of protease digestion of proteins in solution has known disadvantages. These include the step that requires up to 18 h (overnight) incubation to produce peptides in a reasonable amount. Another is the auto-digestion of the enzyme that produces peptides similar to the protein being digested and causes chemical noise that complicates interpretation of results. IMERs resolve these drawbacks by their reduced enzyme-to-substrate ratio and corresponding reduced autolysis of enzyme and enhancement of the digestion efficiency. Moreover, a solid support increases the stability of the enzyme against organic solvents and chemical denaturants from the preparation steps prior to digestion [147]. IMERs in fused silica capillary format have also been coupled with other sample preparation steps to create an on-line system that increases throughput and reproducibility of MS based peptide and protein analysis [148].

There are several options to immobilize enzymes, such as entrapment in polyacrylamide gels or via covalent attachment onto the surface of microbeads and monolithic columns. Attachment of enzymes to the inner walls of open capillaries or microchannels in microfluidic devices has also been reported. The use of monolithic supports offers many advantages, such as ease of fabrication and fast mass transfer and low backpressure of the polymeric support. Rapid conversion of substrates was also observed when using monolithic supports due their large accessible surface area and short diffusion path length. Recent strategies on development of IMERs are focused on increasing the amount of enzyme immobilized, decreasing nonspecific adsorption, and improving enzyme efficiency and stability (Table 3). Improving the hydrophilicity and biocompatibility of the monolithic support can reduce the nonspecific adsorption. Employing mild enzyme immobilization methods can maintain enzyme activity and stability. Using monolithic materials with increased specific surface area can increase the enzyme loading and therefore provide a higher enzyme to substrate ratio. However, one recent study compared two kinds of monolith-based trypsin-based IMERs in terms of monolith properties and digestion performance [149]. It was found that it was not the capacity for trypsin immobilization but the physical properties, such as penetrability and pore distribution of the monolith, that were the factors in digestion performance of IMER. These two monoliths were poly(tetraethoxysiLane-co-3-aminopropyltriethoxysilane) + (poly (TEOS-co-APTES)) monolith and poly(N-acryloxysuccinimide-co-acrylamide-co-ethylene dimethacrylate) + (poly(NASco-AA-co-EDMA)) monolith. The former obtained a higher chromatography

permeability constant (66.7 vs. 4.37) and has more pores and more efficient IMER than the later even less amount of trypsin that was immobilized.

Table 3. Recent strategies in development of IMERs for in glycoprotein analysis.

Strategies	Monolithic Solid Support	Enhanced Features of IMERs	Ref/Year
Use of monolith with better penetrability and pore distribution	Poly (tetraethoxy-silane-co-3-aminopropyl-triethoxysilane) (poly (TEOS-co-APTES)) monolith	More efficient digestion performance than an IMER with higher amount of immobilized trypsin	[149] 2018
Preparation of monolith via thermally induced phase separation (TIPS), resulting in monolith with uniform porosity and high surface area even without using templates and porogens	Poly(glycidyl methacrylate-co-methyl methacrylate) (PGM) monolith	The immobilized pepsin showed better pH and thermal stability compared with free pepsin. Used in online digestion liquid chromatography-mass spectrometry LC-MS and LC-MS/MS systems; larger number of peptides were reproducibly identified compared to those by polystyrene/ divinylbenzene particle (POROS)-based online pepsin column	[150] 2015
Immobilized two enzymes (trypsin and chymotrypsin) for consecutive digestion of proteins	Hybrid monolithic column with SBA-15-NH_2 nanoparticles	Identified 1091 proteins and 5071 peptides in digesting rat liver proteins. Shortened digestion time compared with solution-based consecutive digestion (from 24 h to 94 s)	[151] 2016
Immobilized multiple proteases—trypsin/Lys-C mixture and Lys-N	N-acryloxy-succinimide-co-acrylamide-co-N, N′-methylenebisacrylamide (NAS-AAm-Bis) monolith	Comparable MS signal and protein sequence with in-solution digestion of protein mixture but significantly shortened reaction time (<1 h) and sample loading amount	[152] 2015
Online configuration LC–ESI–MS/MS with serially connected trypsin and PNGase F micro-reactors	Dextran-coated fused silica capillaries	Better sensitivity, efficiency, and speed with reduced potential contamination than with an off-line (in solution) enzyme digestion; greater yield of tryptic peptides produced than in-solution digestion.	[153] 2015
Use of thiol-ene (TE) polymers that have a large excess of functional groups for enzyme immobilization	In-chip thiol-ene (TE) monoliths (anchored in microfluidic channels)	Reversible or covalent irreversible immobilization of PNGase F enzyme, both with good enzymatic activity in deglycosylation of ribonuclease B	[154] 2015
Use of trypsin IMER in glycosylation mapping of a highly glycosylated protein	Enzyme-coupled Sepharose	Identified 42 out of 45 common glycans identified by in-solution digestion; identified more glycans than using pepsin IMER, 2 out of 4 N-glycosylation sites of hCG were identified complementary to pepsin IMER	[155] 2020

A polymer monolith was used as the solid support for covalently immobilizing pepsin to develop an online digestion liquid chromatography-mass spectrometry (LC-MS) system [150]. This poly(glycidyl methacrylate-co-methyl methacrylate) (PGM) monolith was prepared using thermally induced phase separation (TIPS). In TIPS, a polymer solution was prepared at high temperature and then cooled to induce phase separation. A monolith with uniform porosity and high surface area was formed after phase separation without the use of templates and porogens. The produced PGM monolith was then modified with aminoacetal to contain aldehydes (PGM-CHO), to which pepsin enzymes were immobilized by reductive amination. The pH and thermal stability of pepsin was improved upon immobilization. Using the developed LC-MS and LC-MS/MS systems, more peptides were reproducibly identified compared to the number found using a polystyrene/divinylbenzene particle (POROS)-based online pepsin column.

An IMER with combined monolithic enzyme reactors using two enzymes (trypsin and chymotrypsin) was prepared for consecutive digestion of proteins [151]. Trypsin cleaves peptides at the C-terminal side of lysine (K) and arginine (R) residues. Therefore, for proteins with no lysine and arginine residues, such as membrane proteins, digestion efficiency is low. Chymotrypsin cuts specifically at phenylalanine (F), tryptophan (W), tyrosine(Y), leucine (L), and methionine (M), and this complements the cleavage sites of trypsin [156]. The objective was to obtain high-throughput proteolysis of target proteins in complex samples, which was not fulfilled by conventional solution-based digestion done using an enzymatic reactor with one protease immobilized. A previous report already showed that a great increase in sequence coverage and production of more meaningful peptides of targeted proteins could be attained by in-solution digestion with multiple enzymes [157]. However, this method suffered from longer digestion time, protease autolysis, and low

enzyme-to-substrate ratio. In this new bottom-up approach, each individual reactor used hybrid monolithic column with SBA-15-NH_2 nanoparticles embedded with immobilized one protease (trypsin or chymotrypsin). Proteins with different hydrophobicity and molecular weights (bovine serum albumin, carbonic anhydrase, and myoglobin) were used. The best digestion performance was obtained when proteins were digested first by trypsin using a 1:1 trypsin-to-chymotrypsin ratio. In digesting rat liver proteins, 1091 proteins and 5071 peptides were identified. Compared with digestion carried out consecutively in solution, the digestion time of this method was reduced from 24 h to 94 s.

Another IMER with multiple proteases, using trypsin/Lys-C mixture and Lys-N was also developed using a monolithic column [152]. The monolith was prepared using N-acryloxysuccinimide-co-acrylamide-co-N, N′-methylenebisacrylamide (NAS-AAm-Bis) polymerized within silanized 200-µm i.d. fused-silica capillaries. The IMER was used to digest proteins in minutes for delivery to MS for analysis (offline coupling between IMER and MALDI-TOF/TOF and MALDI-LTQ-Orbitrap). Comparable MS signal and protein sequence was observed using this IMER monolith and in-solution digestion of the protein mixture but with a significantly shorter reaction time and lower sample loading amount required. Similar results were obtained in large-scale proteomic studies by enzymatic digestion of mouse serum, yeast, and human cell lysate samples. Online coupling to MS analysis is also feasible, as there were also no extra steps needed for cleaning.

Another online configuration of LC–ESI–MS/MS was developed with serially connected trypsin and PNGase F micro-reactors for digestion and deglycosylation of recombinant human erythropoietin from equine plasma [153]. Microreactors for trypsin digestion were prepared either by immobilizing the enzyme within fused silica capillaries modified by a dextran coating or with capillaries containing a porous monolith. Preparation of this micro-reactor was by a simple, single-step method and required a short fabrication time. A peptide-N-glycosidase F microreactor was also produced and coupled with the trypsin micro-reactor. These microreactors were biocompatible and highly flow permeable, which can provide good enzyme stability and rapid mass transfer. Unique deaminated peptides T5 DAM and T9 DAM from recombinant human erythropoietin were detected by the LC–ESI–MS/MS analysis. The online platform offered better sensitivity, efficiency, and speed with reduced potential contamination as compared with an off-line (in solution) enzyme digestion. Tryptic peptides were produced from digestion in the micro-reactor in a greater yield than obtained by in-solution digestion.

In-chip emulsion-templated monoliths inside microfluidic channels were prepared by mixing TE monomers (tetrathiol and triallyl) and pouring them into polydimethylsiloxane (PDMS) molds before illumination with UV light. After curing, the TE parts were peeled off from the soft PDMS molds to produce in-chip TE monoliths [154]. The resulting in-chip monolith was a microporous network of highly interconnected and size uniform thiol-ene beads anchored securely inside thiol-ene microchannels. Thiol-ene (TE) polymers are currently a popular choice for fabrication of microfluidic devices for bioanalytical applications. A large number of functional groups (thiols or thiol-enes) on the polymer surfaces and available as covalent anchors for attachment of biomolecules can be generated depending on the stoichiometric ratio of initial reactant monomers [158]. Peptide-N-glycosidase F (PNGase F) was immobilized onto the thiol-ene monoliths either reversibly or covalently and irreversibly. The reversible immobilization utilized the cysteine groups on the protein surface to link to free thiol functional groups within the monoliths and form disulfides. The latter utilized free primary amino groups on the protein surface and linked to thiol-ene via click chemistry and L-ascorbic acid linkage. The PNGase F microreactors were highly effective for the deglycosylation of ribonuclease B.

A trypsin IMER was used in glycosylation mapping of a highly glycosylated protein (human Chorionic Gonadotropin hormone, hCG) with analysis done using nano-LC coupled to tandem MS (nanoLC-MS/MS) [155]. hCG is a glycoprotein with four N- and four O-glycosylation sites. Trypsin IMER digestion gave 42 common glycans out of 45 identified by in-solution digestion. When compared to pepsin IMER, more glycans were identified on

a specific glycosylation location, and two out of four N-glycosylation sites of hCG were identified. There are several other approaches in doing glycosylation mapping, such as using glycosidases (e.g., PNGase F) and using Pronase E. However, these approaches are very expensive, and there is possible loss of information if the protein has more than one glycosylation site or if multiple proteins are present in the sample.

6. Conclusions

Glycoprotein analysis aims to elucidate the structure of glycoproteins and the linked glycans to understand how their structure dictates their biological function. The use of monolithic materials as solid support allows more efficient and more convenient digestion, enrichment, and separation processes in glycoprotein analysis. Most of the recent strategies focus on changing the preparation conditions to create better monoliths that can load more ligands with less non-specific binding and therefore achieving the most selective and most efficient separation. The numerous interactions with glycans create a longer list of ligands that can be used in enrichment and separations of glycoproteins and glycopeptides. The effect of column 3D geometry has also been studied to create faster and efficient separation. In the future, monolithic structures could be easily optimized and replicated using 3D printing. Miniaturization of monolithic materials in columns and capillaries are continuously explored to create the most effective high-throughput multidimensional systems. These set-ups use IMERs that allow on-line digestion of glycoproteins and separation of glycopeptides towards detection. Advancement in MS is one of the best contributions in glycoprotein analysis allowing for more effective identification of glycans and glycosylation sites.

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