Nanomaterial Fused Monolithic Microcolumns for Biomolecule Separation

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Achieving a highly pure biomolecule of interest is of great importance and chromatography based techniques are the reliable tools for this challenging task. During the last few decades, monoliths have been explored as alternative chromatography stationary phases for biomolecule separation. In this Minireview, we aim to provide information on different nanomaterials incorporated within the network of monoliths and strategies applied to perform nanomaterial surface functionalization. Subsequently, the applications of “nanomaterials embedded monolithic microcolumns” for separation of target biomolecules such as proteins and peptides with the help of different chromatography principles such as affinity, ionic and hydrophobic is also discussed in detail.

Keywords monolith, nanomaterial, proteins, peptides, separation

Introduction

Chromatography based approaches are widely employed both in academia and industry for selective target biomolecule (proteins, peptides, carbohydrates, etc.) separation from other interfering molecules present in serum, plant extracts, etc.[1-4] However, the technical requirements (tedious packing processes, high back pressures, and the need of frits to hold the resin within the column) of conventional chromatography processes, high back pressures, and the need of frits to hold the material within the microcolumn due to covalent adhesion of material with the column inner wall surface.

Since early 2000s, incorporation of nanomaterials within the confines of monolithic network was initiated to further enhance the separation performance of the monoliths.[16-23] The addition of nanomaterials was achieved either prior to monolith in situ polymerization or immobilized after the monolith is formed, i.e., form chemical bonds with the functional groups present on the monolith surface. The rationale to choose nanomaterials as anchors is due to their impressive inherent physical and chemical properties that enhance the separation performance of the monoliths.

The major objective of this Minireview is to bring out the different nanomaterials incorporated within the monolithic structures and to highlight the biomolecule separation achieved by these new hybrid materials. Table 1 presents the nanomaterials incorporated within monoliths and the ligands used for separation of target analytes.

Gold nanoparticles (Au NP)

The group of Prof. Švec and Prof. Fréchet has extensively worked on various approaches to incorporate Au NP’s within the monolith matrix. In their first study,[20] Au NP’s (15 nm) were immobilized homogeneously on thiol functionalized poly GMA-EDMA (glycidyl methacrylate-co-ethylene dimethacrylate) monolith and were used as ligands to target cysteine functionalized peptides. The adsorption capacity of this monolith for L-cysteine, the model analyte, was found to be 2.50 μmoL/monolith. The monolith adsorbed selectively “His-Cys-Lys-Phe-Leu”. i.e., cysteine containing peptide only and not non-cysteine peptides (Tyr-Gly, Phe-Gly-Phe-Gly, and Tyr-Gly-Gly-Phe-Leu). Although the Au NP monolith has the desired affinity property, it lacked the required hydrophobicity like a conventional reverse phase column to separate peptides. This issue was addressed by fusing the Au NP monolith column with a commercial C18 column (Acclaim PepMap) in a tandem fashion. This strategy has led to an efficient capture of cysteine peptides via Au NP monolith while non-cysteine peptides passed freely and retained by C18 column. In the second study,[23] they introduced various functional groups on Au NP’s present on
the monolith surface both by individual modification and interchangeable functionality between them (Figure 1) by simple chemical reactions. This new strategy has resulted in a diverse Au NP monolith with functional groups like COOH, OH and NH2, which were then directly used as ligands for efficient separation of peptides (Tyr-Gly, Tyr-Gly-Gly, and Tyr-Gly-Gly-Phe-Leu) and proteins (ribonuclease A, cytochrome c, and myoglobin). It is worthwhile to note that Au NP immobilization did not affect the original permeability of the poly GMA-EDMA monolith, i.e., 0.26 MPa/cm. To increase total coverage of Au NP’s on poly GMA-EDMA monolith, they designed a three-step chemical strategy([26]) (1) immobilization of cysteamine on epoxy monolith via ring opening reaction to form cystamine-monolith; (2) treatment with tris(2-carboxylethyl) phosphine has liberated thiol groups present in cysteamine to form thiol-monolith; and (3) immobilization of Au NPs on the thiol-monolith. EDS (energy dispersive X-ray spectroscopy) experimental data indicated that maximum nanoparticle loading (60.6 wt%, no aggregation of nanoparticles) on the monolith was achieved with 40 nm Au NP. In this study, 1-octanethiol and 1-octadecanethiol were the ligands and were immobilized on Au NPs present on monolith. Good protein (ribonuclease A, cytochrome c, and myoglobin) separation was achieved when monoliths were incorporated with 15—30 nm Au NP’s (46.5 wt%—58.4 wt% load) only.

In another study,[27] they prepared a double layered Au NP polystyrene monolithic column to enhance the hydrophilicity for separation of nucleosides. The layered assembly of Au NP’s on monolith was achieved as follows: (i) The native polystyrene monolith with bromine groups was reacted with cystamine followed by tris(2-carboxylethyl) phosphine treatment. This resulted in free thiol groups on the surface; (ii) The first layer of Au NP’s was deposited on the monolith surface by reacting thiol groups with Au NP’s; (iii) An amine molecular layer was created by performing a reaction between the first layered Au NP’s and polyethylene imine (PEI) molecules; (iv) The second Au NP’s layer was finally achieved by reacting free amine groups of PEI with introduction of Au NP’s. For separation of nucleosides (thymine, adenosine, cyidine, cytosine, and guanosine) and peptides (Phe-Gly-Phe-Gly, Val-Try-Val, Gly-Phe, Gly-Leu, Gly-Try, Lys-Val, Gly-Gly-Gly), L-cysteine was used as the ligand on this two-layered Au NP fused monolith.

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**Table 1** Nanomaterials and ligands used for biomolecule separation

| Nanomaterials | Ligands           | Analytes                                | Ref.   |
|---------------|-------------------|-----------------------------------------|--------|
| Au NP         | Au                | Cysteine functional peptides            | [24]   |
| Au NP         | Various ligands   | Peptides and proteins                   | [25]   |
| Au NP         | 1-Octanethiol & 1-octadecanethiol | Peptides and nucleosides            | [26]   |
| Au NP         | Cysteine          | Peptides and nucleosides               | [27]   |
| Au NP         | Erythrina cristagalli | Glycoproteins                            | [28]   |
| Au NP         | 1-Methyl-2-mercaptop-3-butylimidazolium bromide | Nucleosides and nucleic acid bases | [29]   |
| Au NP         | Cysteine          | Glycopeptides                           | [30]   |
| Au NP         | Human α-thrombin aptamer | Human α-thrombin                          | [31]   |
| Au NP         | Au                | Proteins                                | [32]   |
| Iron oxide NP | Iron oxide        | Phosphopeptides                          | [33]   |
| SWNT          | SWNT              | Peptides                                | [34]   |
| MWNT          | MWNT              | Proteins                                | [35]   |
| MWNT          | MWNT-C18          | Proteins                                | [36]   |
| Graphene oxide| Boronate          | Glycoprotein                            | [37]   |
| Latex NP      | Quaternary ammonium | Saccharides                              | [38]   |
| Poly DVB NP & poly EDMA NP | DVB & EDMA | Proteins                                | [39]   |
| Latex NP      | Quaternary ammonium & sulfonate | Proteins                                | [40]   |
| Hydroxyapatite NP | Hydroxyapatite | Phosphopeptides                          | [41]   |
| SiO2/TiO2     | SiO2              | Phosphopeptides                          | [42]   |
| SiO2/TiO2     | Boronic acid      | Glycoproteins                           | [43]   |

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**Figure 1** Schematic diagram of different chemical approaches to modify AuNP’s on the monolith surface.[25]

Alwael et al.[28] immobilized Au NP’s on EDMA monolith pipette tip via Azaclonite chemistry. Subsequent treatment of AuNP’s with 3,3′-dithiodipropionic acid di(N-hydroxysuccinimide ester) resulted in terminal succinimidy1 groups. *Erythrina cristagalli* (ECL) lectin was then immobilized on the monolith and applied for enrichment of glycoprotein desialylated transferrin over non-glycoprotein ribonuclease B. The glycoprotein selectivity of this lectin Au NP-monolith pipette tip was demonstrated in Figure 2 using transferrin spiked *E. coli* cell lysate. Transferrin was only selectively enriched (decrease of peak intensity in chromatogram profile, Figure 2B(b)) from the cell lysate. Figure 2B(c) represents the elution profile of transferrin using galactose as competitive eluting agent in elution buffer.

Lu et al.[29] developed ionic liquid (IL)-Au NP-silica monoliths for capillary electrophorography application. Here, IL (1-methyl-2-mercaptop-3-butylimidazolium bromide) is immobilized on pre-prepared Au NP-silica monolith via the existing thiol molecule in its structure. The presence of IL’s on Au NP-silica monolith surface has resulted in successful separation of thiourea, uridine, guanosine, guanine, and adenine (from a mixture). These molecules are of biological significance and pharmaceutical interest.
Zhang et al.\[26\] developed an efficient system to selectively enrich glycopeptides from a 5 μg digested human plasma sample (without 14 high abundant proteins). Subsequently, an on-line deglycosylation of trapped glycopeptides was done during the elution process. The Au NP immobilized poly GMAPEGDA (glycidyl methacrylate-co-polyethylene glycol diacrylate) monolith was prepared according to Ref. [26] and served as a matrix for both glycopeptide enrichment and on-line deglycosylation. Replacement of EGMA as crosslinker with PEGDA has enhanced the hydrophilicity of the monolith (water contact angle was close to 0). For glycopeptide enrichment, cysteine was the ligand. For on-line deglycosylation, the enzyme PNGase F is the ligand. In both cases, the ligands were immobilized directly on Au NPs present on the monolith surface. The use of cysteine as the ligand helped to perform the glycopeptide enrichment under weak alkaline buffer conditions (10 mM ammonium bicarbonate), which is also a compatible buffer for deglycosylation process. Overall, this study has yielded 196 N-linked glycopeptides that correspond to 122 glycoproteins. In another study,\[31\] they immobilized a “human α-thrombin aptamer (Apt 29) with thiol groups” on the Au NP-GMA-PEGDA monolith. The dynamic binding capacity of the monolith was 277.1 μmol/L. This affinity monolith captured selectively “human α-thrombin” from plasma sample spiked with 1000 fold human α-thrombin and could run over 100 times cycles with no significant loss of capacity.

Curvivan et al.\[32\] designed a two zone monolith capillary system that involves (i) protein trapping by affinity chromatography principle (Au NP was used as ligand) and (ii) separation of eluted proteins by reverse phase chromatography principle. The proof-of-concept was successfully demonstrated using 4 model protein mixture (bovine serum albumin, ribonuclease b, insulin and carbonic anhydrase).

**Iron oxide Nanoparticles (IO NP)**

Krenkova and Foret\[33\] immobilized iron oxide (IO) nanoparticles (average diameter of 19.08 nm) on quaternary amine poly GMA-EDMA monolith. The synthesized IO NP is composed of Fe$_3$O$_4$ and γ-Fe$_2$O$_3$, and they are stable due to the presence of citrate ions. The model molecule, adenosine-5'-nuclease b, insulin and carbonic anhydrase serves as the ligand. In both cases, the ligands were immobilized directly on Au NPs present on the monolith surface. The use of cysteine as the ligand helped to perform the glycopeptide enrichment under weak alkaline buffer conditions (10 mM ammonium bicarbonate), which is also a compatible buffer for deglycosylation process. Overall, this study has yielded 196 N-linked glycopeptides that correspond to 122 glycoproteins. In another study,\[31\] they immobilized a “human α-thrombin aptamer (Apt 29) with thiol groups” on the Au NP-GMA-PEGDA monolith. The dynamic binding capacity of the monolith was 277.1 μmol/L. This affinity monolith captured selectively “human α-thrombin” from plasma sample spiked with 1000 fold human α-thrombin and could run over 100 times cycles with no significant loss of capacity.

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**Carbon based nanotubes**

Li et al.\[24\] prepared single-wall carbon nanotube (SWNT) fused poly VBC-EDMA (vinyl benzyl chloride-co-ethylene dimethacrylate) monolith in a single step in a PEI pre-functionalized silica column. PEI coating was done to generate annular EOF (electroosmotic flow). Incorporation of SWNT has significantly enhanced two properties of the monolith: (i) surface area of the monolith from 7.90 to 11.01 m$^2$/g, and (ii) EOF mobility from 1.95x10$^{-5}$ to 2.53x10$^{-5}$m$^2$.V$^{-1}$.s$^{-1}$ and only a slight increase in the pore size (from 3.26 to 3.35 nm). Separation of the 5 model peptides (methionine encephalin (M), leucine encephalin (L), Val-Tyr-Val (V), angiotensin II (A), and Gly-Tyr (G)) with good retention factor was achieved only if SWNT was present within the monolith structure (Figure 4). The change in the migration order of peptides V and A in presence of SWNT relates to the specific interaction of carbon nanotube with proteins and peptides.

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Minireview

Eric Hilder et al. synthesized SiO$_2$/TiO$_2$ composite monolith (narrow pore size of 3.6 nm, surface area of 115 m$^2$/g) via sol-gel technology and employed SiO$_2$/TiO$_2$ composite as ligand for enrichment of phosphopeptides from tryptic digest of β-casein, α-casein and ovalbumin. In their work, they synthesized a HEMA-EDMA monolith system and incorporated charged NPs ((NR$_3$)$^+$ and SO$_3^-$) into the cryogel. This has enhanced the peak width and peak symmetry along with good separation of all proteins present in the mixture. The results showcase the importance of enrichment process prior to MS analysis.

Hydroxyapatite (HA) nanoparticle

Krenkova et al. incorporated rod shaped HA NPs (average size of 50x150 nm, surface area of 100 m$^2$/g) in poly HEMA-EDMA monolith prior to in situ polymerization. As HA contains both calcium (positive charge) and phosphate (negative charge) molecules, immobilization after monolith polymerization can lead to aggregates. Due to this reason, HA NPs were incorporated to polymer solution followed by polymerization of the composite mixture. With increasing amount of HA NP loading in the monolith, the protein separation (ovalbumin, myoglobin, lysozyme, and cytochrome c) improved but with a significant increase in flow resistance. This HA NP-poly HEMA-EDMA monolith application was studied in two cases: (i) separation of monoclonal antibody complex and (ii) enrichment of phosphopeptides from tryptic digest of β-casein, α-casein and ovalbumin. Overall, this enrichment approach has identified 3 phosphopeptides for β-casein, 11 phosphopeptides for α-casein and 2 phosphopeptides for ovalbumin. Whereas, when analyzed without enrichment process 0 phosphopeptides for β-casein (Figure 5A); 2 phosphopeptides for α-casein (Figure 5B); 11 phosphopeptides for ovalbumin. Whereas, when analyzed without enrichment process 0 phosphopeptides for β-casein (Figure 5A); 2 phosphopeptides for α-casein (Figure 5B); and 0 phosphopeptides for ovalbumin were identified by MALDI-MS. These results showcase the importance of enrichment process prior to MS analysis.

Inorganic nanocomposites

Wang et al. synthesized SiO$_2$/TiO$_2$ composite monolith (narrow pore size of 3.6 nm, surface area of 115 m$^2$/g) via sol-gel technology and employed SiO$_2$/TiO$_2$ composite as ligand for enrichment of phosphopeptides from human serum, non-fat milk and egg white. For example, 11 phosphopeptides were identified from milk samples using SiO$_2$/TiO$_2$ composite in comparison to 5 phosphopeptides using TiO$_2$ material itself and 3 without the use of any material. The obtained data (MS, Figure 6) on human serum sample suggest the impact of SiO$_2$ molecules in the composite.
Figure 6 MS data of Human serum: (A) direct analysis (without enrichment process); (B) enriched using TiO$_2$ material; and (C) enriched using SiO$_2$-TiO$_2$ composite monolith. SEM image of SiO$_2$-TiO$_2$ composite monolith (D) (prepared by J.K.) Phosphopeptides.

Yang et al. prepared a SiO$_2$-TiO$_2$ composite monolithic column functionalized with boronate's in a single step. Here, boronate acts as the ligand to specifically target glycoproteins. The monolith had a well interconnected porous network, pore size of 4.76 nm and a permeability ($K_D = 32.3 \times 10^{14} \text{m}^2$), which is 13 times higher than the conventional packed columns (2.5$ \times 10^{14} \text{m}^2$). The monolith showed excellent specificity to model glycoproteins (ovalbumin and lactoferrin) over nonglycoproteins (bovine serum albumin, myoglobin, and cytochrome c at near physiological conditions (phosphate buffer pH 7). Its affinity to both human and mouse monoclonal antibodies was also demonstrated.

Conclusions

Overall 20 research articles have been published since the inception of nanomaterials within the monolith microcolumns to enhance the separation of biomolecules. The separation of biomolecules was achieved using affinity chromatography, ion exchange chromatography, hydrophilic interaction chromatography, hydrophobic interaction chromatography and in some cases a combination of these chromatography principles. In case of nanomaterials, Au NPs (9 research articles) and carbon based nanomaterials (4 research articles) have been explored more than other nanomaterials. The obtained biomolecule separation using nanomaterials within the confines of monoliths is highly encouraging. The use of nanomaterials in monolithic microcolumns is progressing slowly than what was expected in the early 2000's. We expect the carbon based nanomaterials will be exploited further due to the current trend in the field of nanomaterials and their diverse properties. Also, we can expect a combination of two or more nanomaterials within the monolith to further enhance the separation performance.

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