Enrichment of glycopeptides for glycan structure and attachment site identification

Jonas Nilsson1, Ulla Rüetschi1, Adnan Halim1, Camilla Hesse1, Elisabet Carlsohn2, Gunnar Brinkmalm3 & Göran Larson1

We present a method to enrich for glycoproteins from proteomic samples. Sialylated glycoproteins were selectively periodate-oxidized, captured on hydrazide beads, trypsinized and released by acid hydrolysis of sialic acid glycosidic bonds. Mass spectrometric fragment analysis allowed identification of glycan structures, and additional fragmentation of deglycosylated ions yielded peptide sequence information, which allowed glycan attachment site and protein identification. We identified 36 N-linked and 44 O-linked glycosylation sites on glycoproteins from human cerebrospinal fluid.

Department of Clinical Chemistry and Transfusion Medicine, Institute of Biomedicine, University of Gothenburg, Sahlgrenska University Hospital, Gothenburg, Sweden.
The Proteomics Core Facility, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden.
Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, University of Gothenburg, Sahlgrenska University Hospital, Mölndal, Sweden.

Correspondence to: Jonas Nilsson1 e-mail: jonas.nilsson@clinchem.gu.se
Enrichment of glycopeptides for glycan structure and attachment site identification

Jonas Nilsson1, Ulla Rüetschi2, Adrian Hallin3, Camilla Hesse1, Elisabet Carlson1, Gunnar Brinkmalm1 & Göran Larson1

We present a method to enrich for glycoproteins from unlabelled samples. Sialylated glycoproteins were selectively oxidated, captured on hydrazide beads, trypsinized and released by acid hydrolysis of sialic acid glycosidic bonds. Mass spectrometric fragment analysis allowed identification of glycan structures, and additional fragmentation of deamidylated ions yielded peptide sequence information, which allowed glycan attachment site and protein identification. We identified 35 N-linked and 44 O-linked glycosylation sites on glycoproteins from human cerebrospinal fluid.

Glycosylation is the most frequent and most complex post-translational modification of proteins. Glycans are predominantly glycosidically N-linked to asparagine or O-linked to serine and threonine. N- and O-linked glycans are often terminated with sialic acids. Correct protein glycosylation is important in a wide range of biological processes including protein folding, intracellular sorting, secretion, uptake, and cell and host-microbial recognition. Aberrant glycosylation of N- and O-linked glycoproteins in cancer cells has an important role in tumour growth and metastasis, and glycan analysis may be used as a diagnostic tool. Little is known, however, about the site-specific glycosylation of proteins on a proteomic scale, and for this purpose new glycoproteomic techniques are needed to address the relationship between site-specific glycosylation and their biological function.

N- and O-linked glycans released by PNGase F treatment or by benzylamidation, respectively, can be profiled by mass spectrometry or by fluorescence detection after chromatographic separation. Mild peridate oxidation and online coupling to probes has recently been introduced for the visualization of sialic acids by two-dimensional electrophoresis. Here, we used a new method to enrich glycans on hydrazide beads and determine their structure and attachment site.

As an initial test with a model glycoprotein, we oxidized human transferrin with 2 mM periodate at 0°C and captured it on hydrazide beads. After trypsin digestion we removed the supernatant, containing the tryptic-released peptides, from the beads, and then cleared the immobilized glycopeptides from the resin with 6 M guanidinium hydrochloride, followed by 6 M guanidinium hydrochloride. The washing step was repeated twice, and the glycopeptides were eluted with formic acid. The eluted glycopeptides were subjected to MS/MS analysis, which confirmed the presence of distinct chromatographic peaks corresponding to the masses of Asn352- and Asn236-containing tryptic peptides plus the mass of five hexoses and four N-acetyllactosamines (Hex5HexNac4).

The low-energy collision-induced dissociation fragmentation spectrum (MS2) of the mass to charge ratio (m/z) 1,374.9 peak representing the Asn352-containing glycopeptide and the subsequent spectrum, in which the second-generation fragments (MS3) were acquired (Fig. 1d), showed that the glycopeptides were composed of the complex bi-antennary motif in accordance with published findings (Supplementary Fig. 2). The peptide ion
Sialic acid

From Wikipedia, the free encyclopedia

Sialic acid is a generic term for the N- or O-substituted derivatives of neuraminic acid, a monosaccharide with a nine-carbon backbone. It is also the name for the most common member of this group, N-acetylneuraminic acid (Neu5Ac or NANA). Sialic acids are found widely distributed in animal tissues and to a lesser extent in other species ranging from plants and fungi to yeasts and bacteria, mostly in glycoproteins and gangliosides. The amino group generally bears either an acetyl or glycolyl group but other modifications have been described. The hydroxyl substituents may vary considerably: acetyl, lactyl, methyl, sulfate, and phosphate groups have been found. The term "sialic acid" (from the Greek for saliva, σιαλός/sialon) was first introduced by Swedish biochemist Gunnar Blix in 1952.

Contents

1 Structure
2 Biosynthesis
3 Function
4 See also
5 References
6 Additional images
7 External links

Structure

The two most common sialic acid derivatives are Neu5Ac and Kdn.

N-Acetylneuraminic acid (Neu5Ac) and 2-Keto-3-deoxyxonic acid (Kdn)

The numbering of the sialic acid structure begins at the carboxylate carbon and continues around the chain.

The configuration which places the carboxylate in the axial position is the alpha-anomer.
with glycosidically linked HexNAc (Pep+HexNAc) at m/z 1,351.3 was present in the M5 spectrum, and M5 analysis of this ion resulted in peptide fragmentation (Fig. 1e), which we used in database searching for peptide identification. The m/z 1,351.3 peak (Fig. 1b) originated from the Ane162-containing triptic peptide from beta-2-glycoprotein 1 with a complex biantennary glycan, a contaminant in the transferrin sample. Complex biantennary, fucosylated b- and triantennary glycans were major components, eluting simultaneously with m/z 1,031.5. We analyzed the fragmentation pattern to identify whether fucose was attached to the core or the antenna of the N-linked glycan (Supplementary Fig. 4). We also captured and characterized O-linked glycopeptides from bovine fetrin, another model glycoprotein (Supplementary Fig. 4).

We then applied the static acid capture-and-release strategy to analyze glycopeptides in human cerebrospinal fluid (CSF) samples. We analyzed the supernatant fraction, which mainly contains nonglycosylated triptic-released peptides from captured glycopeptides, by liquid chromatography–MS5 shotgun analysis and identified 84 proteins by database searching (Supplementary Table 1).

For the acid-released glycopeptide fractions (Supplementary Fig. 5), we estimated 4–80% of the M5 fragmented precursor ions to be glycopeptides owing to the presence of the diagnostic ion at m/z 366, [HexHexNAc+4H]+, and because of the characteristic fragmentation patterns at glycosidic linkages.

We characterized N-linked glycopeptides with various workflows and tentatively quantified the relative glycosylation abundance for each N-linked glycopeptide. The glycan structure has been reported previously only for a few of these CSF proteins (Supplementary Table 2). The Pep+HexNAc ion was frequently present in the M5 spectrum and was selected for M5 fragmentation. Mascot searches of the Pep+HexNAc fragment spectra yielded a list of 36 unique N-linked glycosylation sites from 23 glycoproteins, of which all have been reported previously.

Figure 1 | Capture-and-release and mass spectrometry analyses of enriched glycopeptides. (A) Stylated glycopeptides were penicillamine-blocked, captured on hydride beads (Pep+) and tryptic-digested. Tryptic peptides from the captured glycopeptides were released into the supernatant. Glycopeptides were released from the beads by mild formic acid hydrolysis, which selectively cleaves off sialic acids. HexNAc, sialic acid; Gal, galactose; Man, mannose; GlcNAc, N-acetylglucosamine; GalNAc, N-acetyllactosamine; fucNAc, fucose. (B) Reverse phase liquid chromatography–ESI-FTICR mass spectrometry of enriched transferrin glycopeptides. All ions shown are in the [M + Na]+ form and changes are shown in superscript when n = 2. The N-terminal asparagine of the Asn30-containing glycopeptide (n/2, 1,374.9) was deamidated. The N-terminal of the lactosamine core of the Asn42-containing glycopeptide was deaminated (n/2, 1,020.4) or in native form (m/z 1,034.1). (C) MS fragmentation of the Asn30-containing glycopeptide (m/z 1,374.9) showed glycosidic fragmentation. (D) Fragmentation (MS2) at m/z 1,020.4 (in n = 1) was consistent with a complex biantennary structure. (E) MS3 at m/z 1,351.3 (in n = 1, peptide ion with glycosidically linked HexNAc, Pep+HexNAc) gave peptide fragmentation. Apart from the biantennary glycan, triantennary and fucosylated biantennary glycan were also observed for the Asn42- and Asn30-containing glycopeptides. The elution peaks for the individual parent ions were integrated, and an assumption of equivalent enrichment and ionization efficiencies for different glycopeptides, the relative intensities were found to be 48% tri antennary, 30% fucosylated biantennary and 15% triantennary for the Asn42-containing glycopeptide. The Asn30-containing glycopeptide was found to be 49% biantennary, 24% fucosylated biantennary and 26% trisialylated; the absolute identity of mono- and glycopeptides in glycopeptide fragment ions was further confirmed because sequence information cannot be elucidated in the collision-induced dissociation fragmentation of glycopeptides.

The only O-linked glycan we detected from CSF was HexHexNAc–O-Ser/Thr, which is compatible with the core 1 structure Galα13GalNAcα1-O-Ser/Thr (Fig. 2), but could, in principle, also be core 2 structure Galβ14GalNAcα1-O-Ser/Thr for some of these O-linked glycosylation sites. Glycopeptides that contained HexHexNAc could be composed of a HexHexNAc elongated core 1 (for example, Galβ14GalNAcα1–Galβ14GalNAcα1–O-Ser/Thr) or core 2 (Galβ14Galβ14GalNAcα1–O-Ser/Thr) structures, but we found that they were composed of two separate HexHexNAc glycans on different serine or threonine residues because of the sequential loss of two terminal hexose units in the fragmentation spectra (Supplementary Fig. 6) and a lack of glycan fragments exceeding m/z 366. M5 of tryptic O-linked glycopeptides resulted in characteristic fragmentation patterns in which the Pep+HexNAc and peptide ions were the major peaks (Fig. 2).

MS5 of peptide ions yielded peptide fragmentation patterns: Mascot database search of those spectra yielded 44 different O-linked glycosylation sites from 22 different proteins (Supplementary Table 3). Only some of these glycosylation sites have been reported previously. In some cases we deduced the serine or threonine glycosylation site based on peptide fragmentation in the presence of an intact glycan in the sequence (Fig. 2). We noted that a Ser/Thr-X-X-Pro motif, in which X is any amino acid, was present in all but seven of the O-linked glycopeptides; this consensus sequence was consistent with that reported in literature. The brain-specific polypeptide GalNAc transferase GalNAc-T13 has been reported to glycosylate serine or threonine, followed by proline positioned three amino acids away in the sequence of model peptide and may be responsible for the Ser/Thr-X-X-Pro glycosylation patterns we identified for CSF.
Short communication

Synthesis of hydrophilic boronate affinity monolithic capillary for specific capture of glycoproteins by capillary liquid chromatography

Lianbing Ren, Yunchun Liu, Mingming Dong, Zhen Liu*

Key Lab of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, 22 Hankou Road, Nanjing 210093, China

ARTICLE INFO

Article history:
Received 8 July 2009
Received in revised form
30 September 2009
Accepted 6 October 2009
Available online xxx

ABSTRACT

Boronate affinity chromatography is an important tool for specific isolation of cis-diol-containing compounds such as glycoproteins, RNA and carbohydrates. Boronate functionalized monolithic capillaries have been recently developed for specific capture of cis-diol-containing small biomolecules, but the apparent hydrophobicity of the columns prevents their specific capture of glycoproteins. In this paper, a hydrophilic boronate affinity monolithic capillary was prepared by in situ free radical polymerization, using 4-4' vinylphenyliboronic acid (VPGA) and N,N'-methylenebisacrylamide (MBA) as functional monomer and cross-linker, respectively. The prepared poly(VPGA-co-MBA) monolithic capillary exhibited uniform open channel network and high density of accessible boronic acid. Due to the utilization of hydrophilic cross-linker, the prepared column was hydrophilic, allowing for specific capture of glycoproteins.

© 2009 Elsevier BV. All rights reserved.

1. Introduction

Glycoproteomics is an important frontier in life science researches nowadays. The first step of glycoproteomic analysis is the isolation of glycoylated proteins from the remainder of the protein. Boronate affinity chromatography (BAC) [1-5], which relies on covalent reaction between cis-diols and boronate ion, has been developed for trapping of glycosylated proteins. The principle is based on the chemistry that boronic acids form covalent bonds with cis-diols to generate five- or six-membered cyclic esters in a basic aqueous media while the esters reversibly dissociate once upon changing the media to an acidic pH. Zhang et al. [6,7] established proteomic methods for profiling of nonenzymatically glycosylated proteins and peptides by combining conventional boronate affinity chromatography with LC-MS. Sparber et al. [8-10] proposed a novel format of boronate affinity chromatography, boronate affinity magnetic microextraction, for specific capture of glycoproteins and glycopeptides, offline coupled with MALDI-MS for structural identification.

HPLC has been the standard separation platform for coupling with MS in the well-established shotgun approach. Monolithic capillaries-based nano-flow HPLC has drawn increasing attention in proteomic analysis recently [11-23]. Monolithic capillaries feature several significant advantages over regular columns. Monolithic beds can be described as an integrated continuous porous separation media without interparticle voids [23]. Commercially available boronate matrices are mainly in gel forms and therefore unsuitable for HPLC, not to mention nano-flow HPLC. Recently, Potier et al. [24] reported boronate functionalized monolithic column synthesized by post modification on two base polymer monoliths. The monolithic columns were evaluated with the separation of small molecules while no proteins' separation was performed. Recently, we [25] reported boronate affinity monolithic column synthesized by free radical polymerization, poly(4-vinylphenyliboronic acid-co-ethylene glycol dimethacrylate), abbreviated as poly(VPGA-co-EDMA). More recently, we [26] proposed a novel ring-opening polymerization approach for facile synthesis of boronate functionalized monolithic capillary that can capture cis-diol-containing small molecules under neutral pH condition. Both types of monolithic columns, especially the former one, exhibited noticeable reversed-phase retention. To suppress reversed-phase retention, organic solvent is required to be added to the mobile phase. However, for protein samples, the presence of organic solvent increases the risk of protein denaturation and precipitation. Therefore, hydrophilic boronate affinity monolithic capillaries are highly desirable for proteomic samples.

In this study, we synthesized a hydrophilic boronate functionalized polymeric monolithic column by free radical polymerization through substituting the hydrophobic cross-linker EDMA with a hydrophilic one, N,N'-methylenebisacrylamide (MBA). MBA is a hydrophilic bifunctional compound that has been widely used as a cross-linker in the preparation of biocompatible stationary phases.
to an acidic mobile phase (pH 2.7), cation exchange was eluted. These results confirmed the boronate affinity of the monolithic column.

3.4. Reversed-phase retention

The hydrophobicity of the prepared monolithic column was evaluated with isocratic separation of a homologous series of alkyl benzenes, including benzene, toluene, ethyl benzene and propyl benzene, on the column. Triacon was used as the void time marker. Retention factors of the alkyl benzenes on the poly(VPBA-co-MBA) and poly(VPBA-co-EDMA) columns under otherwise identical conditions were measured, as compared in Fig. 4. It can be seen that the retention factors of alkyl benzenes on poly(VPBA-co-MBA) were decreased by 70% as compared with those on the poly(VPBA-co-EDMA) monolithic column. These results suggest that the hydrophobicity of the poly(VPBA-co-MBA) monolith was significantly reduced by utilization of the hydrophilic cross-linker MBA.

3.5. Specific capture of glycoproteins

The main designed function of the boronate affinity monolithic columns is specific capture of glycoproteins. Although the poly(VPBA-co-EDMA) monolith allowed for capture of glycoproteins such as HRP, some non-glycosylated proteins such as BSA exhibited strong adsorption due to reversed-phase retention (data not shown). Such non-specific adsorption is detrimental. It not only degrades the specificity of the boronate affinity column but also reduces its reproducibility and lifetime. For small molecules, this issue can be overcome by adding a certain amount of organic solvent into the loading and elution buffers. For proteins, however, the presence of organic solvent may lead to denaturation and even precipitation of proteins trapped by the column. Therefore, capturing glycoproteins and meanwhile excluding nonglycosylated proteins under aqueous loading and elution buffers are the required capabilities for the specificity. To test the specificity, two typical glycoproteins, including HRP (pI 3.0−9.0) and lactoperoxidase (pI 6.0−9.0), were selected as test molecules while four nonglycosylated proteins, BSA (pI 4.7), lactoglobulin (pI 5.0), myoglobin (pI 6.8, 7.2) and cytochrome C (pI 10.0−10.5), were used as control.

Since the boronic acid groups on the monolithic bed are negatively charged at basic pH, it is essential to suppress electrostatic interactions between the negatively charged monolithic bed and proteins. It was found that when the salt concentration (ammonium acetate) was lower than 200 mM, HRP could not be captured by the column. Therefore, 250 mM ammonium acetate was used as the loading buffer. As shown in Fig. 5, HRP and lactoperoxidase were captured by the boronate functionalized monolithic column. However, the four non-glycosylated proteins were non-retained on the monolithic column, eluted at the void time (indicated by the baseline disturbances). Thus, specific capture of glycoproteins by the poly(VPBA-co-MBA) monolithic column was demonstrated.

The reason for the different elution times and peak shapes for HRP and lactoperoxidase is unclear at present. It should be related to the properties of the proteins but the molecular size. The two proteins are quite different in their molecular weight, 44 and 87 kDa for HRP and lactoperoxidase, respectively. However, they were eluted directly without any affinity from the capillary under isocratic elution with acetic acid (data not shown). If there were any size effect (such as size exclusion), there should have been apparent difference in the retention time under isocratic elution. Therefore, it seems clear that molecular size was not the reason, at least not the main reason, for the delayed elution time and tailing peak shape for HRP under the stepwise gradient elution.

4. Conclusions

A new hydrophilic boronate affinity monolithic capillary was synthesized. The prepared capillary exhibited uniform open channel network and high accessible ligand density. Due to the hydrophilic cross-linker MBA, the prepared monolithic column was hydrophilic and biocompatible, allowing for specific capture of glycoproteins with aqueous mobile phase. As the elution in BAC is carried out with an acidic mobile phase, generally acetic or formic acid, nano-flow HPLC based on boronate affinity monolithic capillary has very good compatibility to MS. Therefore, it can be a promising tool for glycoproteomic analysis.

Acknowledgements

We gratefully acknowledge the financial support of the General Grant (No. 200793001X) from the National Natural Science Foundation of China, the Key Grant of 973 Program (No. 2007CB914010), the international Science & Technology Cooperation Grant (No. 2008DFA01810) from the Ministry of Science and Technology of
Poly[hydroxyethyl acrylate-co-poly(ethylene glycol) diacrylate] Monolithic Column for Efficient Hydrophobic Interaction Chromatography of Proteins

Yuanyuan Li,1 H. Dennis Tolley,2 and Milton L. Lee3,4

Department of Chemistry and Biochemistry and Department of Statistics, Brigham Young University, Provo, Utah 84602

Rigid poly[hydroxyethyl acrylate-co-poly(ethylene glycol) diacrylate] monoliths were synthesized inside 75 μm i.d. capillaries by one-step UV-initiated copolymerization using methanol and ethyl ether as porogens. The optimized monolithic column was evaluated for hydrophobic interaction chromatography (HIC) of standard proteins. Six proteins were separated within 20 min with high resolution using a 20 min elution gradient, resulting in a peak capacity of 54. The effect of gradient rate and initial salt concentration on the retention of proteins were investigated. Mass recovery was found to be greater than 90%, indicating the biocompatibility of this monolith. The monolith was mechanically stable and showed nearly no swelling or shrinking in different polarity solvents. The preparation of this in situ polymerized acrylate monolithic column was highly reproducible. The run-to-run and column-to-column reproducibilities were less than 2.0% relative standard deviation (RSD) on the basis of the retention times of protein standards. The performance of this monolithic column for HIC was comparable or superior to the performance of columns packed with small particles.

Hydrophobic interaction chromatography (HIC) is a valuable technique for the separation and purification of proteins under non-denaturing conditions, which was pioneered by Parikh et al. and Hjerten.1,2 Principles of HIC involve weak hydrophobic interactions of a protein with a moderately hydrophobic ligand distributed on the stationary-phase matrix. This interaction is promoted through the use of a mobile phase containing high salt concentration, such as sodium sulfate, ammonium sulfate, or sodium chloride. The separation is usually achieved by first using an initial high salt concentration that enhances hydrophobic interaction by removing water from the vicinity of the protein surface, and then the retained proteins are eluted in order of increasing hydrophobicity either in an isocratic manner or, more generally, by a descending salt gradient that allows the proteins to rehydrate selectively. This is in sharp contrast to reversed-phase chromatography (RPC) where organic solvents and acidic conditions are used for sample elution, which tends to promote protein denaturation. While both HIC and RPC are based on hydrophobic interaction with the stationary phase to effect separation, an HIC stationary phase differs from RPC mainly in three ways: ligand hydrophobicity, ligand density, and hydrophobicity of the column matrix. Compared to RPC, HIC columns usually have ligands that are less hydrophobic and have lower ligand densities, and the polymeric matrix is relatively hydrophilic. Consequently, HIC is less denaturing and allows elution with entirely aqueous eluents rather than organic solvents.

A number of packing materials have been developed for HIC separation,3–14 while materials in the form of a continuous bed or monolith have been fewer. Application of monoliths as chromatographic phases was introduced in the 1990s.15 As an alternative to packed columns, monolithic columns have received increasing interest because of advantages such as low back-pressure, fast mass transfer, and simple preparation. Excellent reviews have appeared describing the preparation of polymer monoliths and their applications in liquid chromatography.16–18 HIC applications of polymer monoliths are much less widespread than, for example, RPC or ion-exchange chromatography (IEC). Recently, Zhang et al. reported a poly(Vinpropyrolacrylamide) grafted polymer monolith for HIC separation of proteins,19 in which poly(Vinpropyrolacrylamide) was grafted onto a poly(chloromethylstyrene)-

---

1 To whom correspondence should be addressed. Email: tly@byu.edu
2 Department of Chemistry and Biochemistry.
3 Department of Statistics.
4 Department of Life Sciences.
under 3.0 M initial (NH₄)₂SO₄ concentration, and a distorted peak is observed. A small peak between cytochrome c and myoglobin was periodically observed, which most probably originated from degradation of protein samples, such as oxidation/reduction of cytochrome c.

The reason why these three polymerization solutions were chosen to prepare columns is because they yield monoliths with similar backpressures. We already pointed out that the addition of HEA increases the polarity of the HEA/PEGDA copolymer. The use of methanol in the porogen mixture leads to a greater percentage of micropores when the HEA concentration is higher and vice versa. At the same time, an increase or decrease in cross-linker to monomer ratio can raise or reduce the pressure drop. Although 85 wt% ratio methane: ethyl ether yielded a monolith with much lower back-pressure than 60 wt% ratio when the weight ratio of HEA to PEGDA was 1:1, it produced a column with similar flow resistance when the weight ratio of HEA to PEGDA was 13:1 because of the decrease in HEA. When the HEA to PEGDA ratio was increased to 13:1, the effect of methanol was mitigated by the decrease in cross-linker. Therefore, these three columns exhibited a similar pressure drop. Despite this, column B showed the best performance. SEM images of the three columns indicate that column B is more homogeneous than the other two. Binding capacity is another important property which is discussed later.

**Effect of Elution Gradient on the Elution of Protein Standards.**

The effect of gradient rate on protein retention and resolution were examined using column B. As shown in Figure 4, for all gradient rates, the proteins were eluted as sharp peaks, indicating that there was little nonspecific protein adsorption when using this HEA/PEGDA monolith. The performance is comparable or superior to the performance of HIC packed columns. Essentially, baseline separation is achieved even with a short gradient time of only 5 min. In this case, the gradient volume corresponds to only 2.1 column volumes, while the 10, 15, and 20 min gradients represent 4.2, 6.4, and 8.6 column volumes, respectively. Resolution values for ribonuclease A and bovine serum albumin and α-chymotrypsinogen A were calculated for each gradient rate and listed in Table 2. Peak capacities were calculated by dividing the gradient time by the average peak width of peaks 2–6. The peak widths were obtained directly from integration using Chromatography software. The results indicate that the shallower gradients afforded better resolution and higher peak capacity, with the greatest improvement arising from increasing the gradient time from 5 to 10 min. As the gradient became more shallow, the degree of improvement became smaller.

The run-to-run reproducibility of the poly(HEA-co-PEGDA) column was quite good. Four runs carried out on separate days using conditions as in Figure 4D, the relative standard deviation (RSD) of the retention times for proteins 2–6 were 0.73, 0.77, 0.67, 0.25, and 0.17%, respectively. These data not only demonstrate good reproducibility but also indicate the stability of the monolithic column. Reequilibration of the column was readily achieved with starting buffer; approximately two column volumes for approximately 6 min were sufficient. Column-to-column reproducibility was also measured, and RSD values (n = 3) of retention times for proteins 2–6 were 2.0, 0.98, 0.74, 0.61, and 1.1%, respectively.

![Figure 4](image_url) **Figure 4.** HIC of protein standards with different gradient rates of (A) B, (B) C, (C) 15, and (D) 20 min. Other conditions are the same as in Figure 3B.

**Table 2. Resolution and Peak Capacity of Protein Standards Separated Using Different Gradient Times**

<table>
<thead>
<tr>
<th>gradient time (min)</th>
<th>resolution</th>
<th>peak capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.98</td>
<td>50</td>
</tr>
<tr>
<td>10</td>
<td>3.17</td>
<td>64</td>
</tr>
<tr>
<td>15</td>
<td>4.03</td>
<td>48</td>
</tr>
<tr>
<td>20</td>
<td>3.98</td>
<td>54</td>
</tr>
</tbody>
</table>

* Resolution of peaks 5 and 6, α-chymotrypsin and α-chymotrypsinogen A. * Resolution of peaks 3 and 4, ribonuclease A and α-chymotrypsinogen A.

* Peak capacity = time of gradient/average peak width.

**Effect of Initial Salt Concentration on the Retention of Protein Standards.** In HIC, selectivity and resolution can be modulated by adjusting stationary-phase variables such as ligand type or ligand density and/or by adjusting mobile-phase variables such as...
Review

Biocompatible polymeric monoliths for protein and peptide separations

The concept of biocompatibility with reference to chromatographic stationary phases for separation of biomolecules (including proteins and peptides) is introduced. Biocompatible is a characteristic that indicates resistance to nonspecific adsorption of biomolecules and preservation of their structures and biochemical functions. Two types of biocompatible polymeric monoliths [i.e., polyacrylamide- and poly(meth)acrylate-based monoliths] used for protein and peptide separations are reviewed in detail, with emphasis on size exclusion, ion exchange, and hydrophobic interaction chromatographic modes. Biocompatible monoliths for enzyme reactors are also included. The two main synthetic approaches to produce biocompatible monoliths are summarized, i.e., surface modification of a monolith that is not inherently biocompatible and direct copolymerization of hydrophilic monomers to form a biocompatible monolith directly. Integration of polyethylene glycol into the poly(meth)acrylate monolith network is becoming popular for reduction of non-specific protein interactions.

Keywords: Monoliths | Biocompatibility | Non-specific adsorption | Proteins | Peptides | Polyethylene glycol | HPLC | Capillary column

Received: July 4, 2009; revised: August 25, 2009; accepted: August 25, 2009

DOI: 10.1002/jssc.2009001475
Review

Porous polymer monoliths: Amazingly wide variety of techniques enabling their preparation

Frantisek Svec a, b, *  

a The Molecular Foundry, E. O. Lawrence Berkeley National Laboratory, MS 67861-10, Berkeley, CA 94720-8190, USA  
b Department of Chemistry, University of California, Berkeley, CA 94720-1460, USA

ABSTRACT

The porous polymer monoliths went a long way since their invention two decades ago. While the first studies applied the traditional polymerization processes as at that time well established for the preparation of polymer particles, creativity of scientists interested in the monolithic structures has later led to the use of numerous less common techniques. This review article presents vast variety of methods that have meanwhile emerged. The text first briefly describes the early approaches used for the preparation of monoliths comprising standard free radical polymerizations and includes their development up to present days. Specific attention is paid to the effects of process variables on the formation of both porous structure and pore surface chemistry. Specific attention is also devoted to the use of photopolymerization. Then, several less common free radical polymerization techniques are presented in more detail such as those initiated by γ-rays and electron beam, the preparation of monoliths from high internal phase emulsions, and cryogels. Living processes including stable free radicals, atom transfer radical polymerization, and ring-opening metathesis polymerization are also discussed. The review ends with description of preparation methods based on polycondensation and polyaddition reactions as well as on precipitation of preformed polymers affecting the monolithic materials.

© 2009 Published by Elsevier B.V.
Integrated Platform for Proteome Analysis with Combination of Protein and Peptide Separation via Online Digestion

Huiming Yuan, Liu Zhang, Chunyan Hou, Guizhu Zhu, Yingyan Tuo, Zhen Liang, and Yukui Zhang

Key Laboratory of Separation Science for Analytical Chemistry, National Chromatographic R & A Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China and Graduation School of Chinese Academy of Sciences, Beijing 100039, China

An integrated platform with the combination of protein and peptide separation was established via online protein digestion, by which proteins were first separated by a microcolumn packed with mixed weak anion and weak cation exchange (WAX/WCS) particles under a series of salt steps, online digested by a trypsin immobilized microenymatic reactor (IMER), trapped and desalted by two parallel C8 precolumns, separated by microreversed-phase liquid chromatography (μRPLC) under a linear gradient of organic modifier concentration, and finally identified by electrospray ionization-MS/MS (ESI-MS/MS). To evaluate the performance of such a platform, a mixture of myoglobin, cytochrome c, bovine serum albumin (BSA), and o-casein, with mass ranging from 25 ng to 2 μg, was analyzed. Compared to the methods by offline protein fractionation and shotgun based strategy, the analysis time, including sample preparation, digestion, desalting, separation, and detection, was shortened from ca. 30 to 5 h, and cytochrome c with abundance of 25 ng could be identified with improved sequence coverage. Furthermore, such an integrated platform was successfully applied into the analysis of proteins extracted from human lung cancer cells. Compared with the results obtained by the shotgun approach, the identified protein number was increased by 30%. All these results demonstrated that such an integrated approach would be an attractive alternative to commonly applied approaches for proteome research.

"Top-down" and "bottom-up" approaches are two main analytical strategies for proteome research. By the "top-down" approach, intact proteins are usually separated and identified by mass spectrometry (MS). Since the accurate mass of proteins could be obtained, this approach might be advantageous for identifying translational start and stop sites, mRNA splice variants and post-translational modifications (PTMs) of expressed gene products. By the "bottom-up" approach, proteins are first digested into peptides and then separated by multidimensional chromatography, and finally identified by MS/MS. Since the separation of peptides is much easier than that of proteins, the "bottom-up" strategy has recently become popular in proteome research.

However, both of the above-mentioned strategies have some shortcomings. By the "top-down" strategy, protein processing is challenging due to typical complexities associated with intact protein purification. By the "bottom-up" strategy, the simultaneous separation of all peptides digested from the whole proteome brings great challenges not only to 2D-high performance liquid chromatography (2D-HPLC) separation but also to the identification by MS/MS. To solve this problem, offline protein prefractionation was performed before peptide separation by HPLC, to decrease the complexity of samples. However, most of the offline approaches suffer from sample loss, time-consuming operation, and difficulty in automation. Therefore, a novel approach combining online protein separation, digestion, peptide separation, and protein identification might be a good solution.

To achieve rapid online digestion of proteins, one key product for the integration of protein processing and peptide analysis has been immobilized enzymatic reactors (IMER), in which proteases...
Figure 4. Base peak chromatograms of a four-protein mixture analyzed by the integrated platform. Experimental conditions were shown in the Experimental Section.

µPLC-ESI-MS/MS. It was shown in Figure 2b that cytochrome c digested within a short residence time could be identified with sequence coverage over 25%, with at least four peptides matching. The enhanced enzymatic activity with trypsin immobilized on supports might be caused by the increased enzyme concentration in fixed space and by the decreased possibility for the autodigestion of enzyme.

Although all these results demonstrated that our developed acrylo polymer particles based IMER was competent in the integrated platform to couple proteins and peptide separation, further effort should be made to reduce protein residual and improve the digestion of low abundance proteins.

Construction of Integrated Platform. The schematic diagram of the integrated platform with the combination of online protein separation, digestion, peptide separation, and protein identification was shown in Figure 3a. During the operation, proteins were first injected onto a WAX/WXC column by switching valve 1 and then separated under a series of salt steps. Subsequently, each fraction eluted from the WAX/WXC microcolumn was online digested by an IMER. Finally, the digests were analyzed by µPLC-MS/MS. In our experiments, to ensure the time synchronization of proteins and peptide separation, two C8 precolumns were used to in turn capture the protein digests by the control of valve 2, as shown in Figure 3b, to ensure the analysis time of peptides by µPLC-MS/MS equal to that of proteins separated by a WAX/WXC microcolumn.

Evaluation of Integrated Platform. To evaluate the performance of our integrated platform, a four-protein mixture, including 25 µg of cytochrome c, 500 µg of myoglobin, 2 µg of BSA, and 2 µg of α-casein, with pH ranging from 4 to 9, was separated by mixed-mode µIEC into four fractions by four salt steps, including 10, 200, 350, and 1000 mM ammonium acetate, and each fraction was further online digested by IMER, followed by µPLC-MS/MS analysis, as shown in Figure 4. By database searching, the sequence coverages for myoglobin, cytochrome-c, BSA, and α-casein were respectively 41 ± 36, 41 ± 34, 41 ± 26, and 36 ± 4% in triplicate runs, as shown in Table 1. In addition, the RSDs of the retention time of identified peptides found to be below 2.9% (as shown in Table S2 in the Supporting Information). All these results demonstrate the good reproducibility of the established integrated platform.

To compare the performance of integrated platform with other commonly applied methods, the same four-protein mixture was also analyzed by offline protein preextraction by µIEC, in-solution digestion, and peptide analysis by µPLC-MS/MS under

| Table 1. Sequence Coverage of Four Proteins Obtained by Three Different Methods in Triplicate Runs |
|-------------------------------------------------|-------------------------------------------------|-----------------------------------------------------------------|
|        | integrated | offline | shotgun               |
| protein | approach    | approach | approach              |
|         | (h)         | (h)      | (h)                  |
| myoglobin (2 µg) | 0 - 1.5 | 0 - 1.5 | 0 - 1.5 |
| cytochrome c (25 µg) | 0 - 1.5 | 0 - 1.5 | 0 - 1.5 |
| bovine serum albumin (2000 µg) | 0 - 1.5 | 0 - 1.5 | 0 - 1.5 |
| α-casein (1000 µg) | 0 - 1.5 | 0 - 1.5 | 0 - 1.5 |

*Peptides were considered positive if Xcorr values were higher than 1.5, 2.2, and 3.75, respectively, for singly, doubly, and triply charged peptides, and Xcorr cutoff values were 2.0.1. Database: bovine, bacta, and homo./* The total time for analysis of the four-protein mixture, including digestion, LC separation, and MS detection.
Review

Plucking, pillaging and plundering proteomes with combinatorial peptide ligand libraries

Pier Giorgio Righetti¹,², Egisto Boschetti³, Alberio Zanella³, Elisa Fasoli³, Aritilio Citterio⁴

¹ Department of Chemistry, University of Milano and Chemical Engineering "Claude R exact", Politecnico di Milano, Via Marzabotto 2, Milano 20133, Italy
² Rea-Roth Laboratorio, 3, Boulevard E. Paoloni, 9240 Shanghai, Tianjin, People's Republic of China
³ Hum Logic, Via Cipolla 5, 20132 Milano, Italy
⁴ AstraZeneca, 26090 Via G. Finzi, Milano, Italy

ABSTRACT

Recent developments in the technique of combinatorial peptide ligand libraries, for enhancing the visibility of low-quantity proteomes, are reviewed here. Novel extraction systems, allowing essentially complete proteome recovery in a single step, are reported here, particularly, on-bead elution with 3-5% boiling sodium dodecyl sulphate (SDS) or in one-phase CHAPS with either 40 mM Tris or 25 mM cysteic acid. New capturing systems are also discussed, in particular, although capturing at pH 7.2 is physiological saline has always been recommended, it is shown that capturing at pH 7.2 and alkaline (pH 8.5) values substantially increase the total captured protein population. Some examples of detection of novel proteins by the described methodology are also discussed. In particular, in the case of venom proteomes, where essentially all components had been detected and fully described by conventional means, the application of the ligand library technology allowed the discovery of two, previously unreported, trace enzymes necessary for the maintenance of the native structure of venom components, namely peptidyl analog cyclohexyl cyclase. In the case of red blood cell (RBC) cytoplasmonic proteomes, where a grand total of 1570 components of the 26 minor proteomes have been identified, these findings allowed to unravel the genetic defect of a rare RBC disease, called congenital dyserythropoietic anaemia type II. The mutations are located in the SEC238 gene coding for the SEC238 protein, detected for the first time in the SEC238 protein thanks to the peptide capturing technology.

© 2009 Elsevier B.V. All rights reserved.

Contents

1. Prologue ........................................................................................................................................................................ 00
2. Introduction ................................................................................................................................................................. 00
3. General properties and behaviour of hexapeptide ligand libraries ............................................................... 00
4. Novel elution protocols ............................................................................................................................................... 00
5. Proteome capture at different pH values .......................................................................................................................... 00
6. Examples of some biological applications ................................................................................................................... 00
   6.1. Snake venoms ......................................................................................................................................................... 00
   6.2. Newest insights on the red blood cell cytoplasmonic proteomes ........................................................................ 00
7. Discussion ..................................................................................................................................................................... 00
Acknowledgements ............................................................................................................................................................. 00
References ............................................................................................................................................................................ 00

1. Prologue

As odd as it sounds, pillaging, plucking and plundering (PPP) has been the favourite past time of all past "civilizations", to the point that the history of human kind is the account of all disasters provoked by infinite wars and the ensuing PPPs perpetrated by the winnors onto the losers (unfortunately it does not seem that today we are doing any better!). Perhaps one of the most chilling PPP events took place in 1527 in Rome, and in fact it went down to history as "il sacco di Roma" (the sack of Rome): it is worth recalling some of the episodes here. In the 16 century Europe was shaken on its foundations by some major events, among which the fierce
somewhat possible with so-called "depletion" methods, but even more importantly because it rendered possible the concentration of low- and very-low-abundance species. The interest of such a possibility is large not only for checking the function of rare species present in a given extract or cell organelle, but also for seeing if the genome is still active for proteins that are supposed to be repressed in their expression after the fetal stage. An additional benefit is to find markers of diagnostic interest as well as markers to follow the efficacy of a therapeutic treatment or to classify populations between responders and nonresponders or even fatal outcome. The possibility of detecting traces of biomarkers permits to diagnose misregulated expressions at earlier stages than with current methods.

4.1 Principle and working mechanism

The most satisfactory, but simplistic, explanation about how a solid phase combinatorial peptide ligand library works for the decrease of the dynamic concentration of protein components from a current biological extract is based on the absolute specificity of a peptide bead for a single protein associated with a binding capacity saturation phenomenon. This principle is explained quite extensively in the seminal paper of Thulliez et al. [23] and then completed in a few other published papers [56-58]. Clearly if each bead of the solid phase peptide library is saturated with a single protein, at the issue of the process all proteins would be collected as a mixture of equally concentrated proteins. Figure 1 summarizes the working scheme.

A survey of the literature of peptide libraries and their interaction with proteins first claimed that peptide beads could be extremely specific for given proteins. On this basis several authors published the use of peptide libraries as a source of affinity ligands for protein purification.

![Diagram](image)

**Figure 1.** Schematic representation of the reduction of dynamic concentration range using combinatorial ligand libraries. Each bead ("a", "b", and "c") carries a single type of ligand and interacts with one protein species (A, B, and C). Proteins in excess (A), compared to bead binding capacity, remain unbound and thus are eluted during bead wash. (A) Situation where one protein (A) is in excess compared to binding capacity of the ligand bead "a". Other proteins (b and c) are totally adsorbed, but they are not concentrated after the treatment because bead binding capacity was not saturated. (B) Situation where all proteins (A, B, and C) are in excess compared to binding capacity of all beads. The resulting sample is reduced in the dynamic protein concentration range.
Figure 6. MS analysis using ProteinChip arrays of different biological samples before (left column) and after treatment with a hexapeptide library (right column). The spectra window focuses on low molecular masses. From “A” to “F” are, respectively, urine proteins (thiourea-urea-CHAPS solution eluate and IMAC-Cu”” array); serum proteins (acidic urea eluate and CM10 array); human bile proteins (thiourea-urea-CHAPS eluate and IMAC-Cu”” array); egg white proteins (acidic urea eluate and IMAC-Cu”” array); human cerebrospinal fluid proteins (acidic urea eluate and CM10 array); saliva proteins (acidic urea eluate and CM10 array).

Figure 7. Venn diagrams obtained by superimposing identified proteins from four biological extracts before (C”) and after treatment with either an amino-terminal library (C”) or a carboxylated hexapeptide library (C”). (A) Red blood cell lysate. (B) Chicken egg white. (C) Human platelet extract. (D) Human cerebrospinal fluid. Numbers inside the circles are the counts of unique proteins identified; numbers outside the graphics represent exclusive proteins found in each analyzed group or common proteins (common areas between circles).

ability to detect novel, unexpected proteins that are naturally present but probably in concentrations below the sensitivity of current instruments.

The exploration of the platelet proteome after treatment by the peptide ligand libraries, followed by 2-DE and MS analysis, allowed expanding the list of known proteins with 147 undescribed gene products (29). Using two different libraries, it was also possible to distinguish proteins captured by an amino terminal peptide library from proteins that were captured by a carboxylated library. In spite of a large redundancy, the second library added a nonnegligible number of proteins, most of them having a relatively alkaline pl. Among proteins found there were a number of proteins not described in the platelets literature such as for example: PMRase, signalosome subunit 2a, LMW-PTP, reticulon-4, and nucleosome-assembly protein-1. In addition other proteins that are either functionally expected in platelet lysates but never reported or known by just their biological activity were Rho-GTPase-activating protein 18, PAF-acetyl hydrolase, and Gal/GalNAc. Interestingly, several proteins from the same signaling pathway (phospho-Tyr signalosome) were also detected, such as the hematopoietic cell-specific LYN, the lymphocyte cytoplasmic proteins 2 (SLP76) and the linker for activation of T-cells (LAT). As in serum and urine, about 18% of normally detectable proteins were neither recognized nor captured by the peptide libraries and hence not detected after the treatment.

Bile fluid is another material of biological source that has been treated with the ligand library and then analyzed to identify the protein composition compared to the nonproteinated material (31). Bile contains a large amount of lipid-like products that needs to be eliminated first to prevent possible bead clogging and reduction of properties of protein-peptide interaction. The initial material comprised 141 gene prod-

© 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim
www.proteomics-journal.com
No side by side comparison is published between narrow IPG and reduction of dynamic concentration range using peptide libraries. These methods are so different from each other that comparison may not be the most objective way to judge their performance. It could however be anticipated that these two methods could be associated for a better detection of low abundance species: the one being used to enhance the presence of low abundance species, and the other allowing to detect proteins that are very similar in their pI.

4.6 Review of published data

Most published data on the use of combinatorial peptide ligands focus on the identification of components of proteomes by taking advantage of the concentration of low-abundance proteins that are rarely described using classical sample pretreatment methods. The most important proteomes investigated are urine, human serum, platelets, lymphocytes, bile fluid, red blood cell lysate, chicken egg white. What is common from these studies is the unambiguous detectability of a much larger number of proteins by using both 2-DE and MS. For instance, the number of spots counted after 2-DE analysis was multiplied by a factor of seven (from 115 to 790 spots for human serum proteins). In all cases new proteins were described for the first time as present in the treated sample. Table 4 reports the number of proteins found before and after treatment for several biological samples. The number of MS signals found after the use of peptide libraries was proportionally larger for masses below 10 kDa than for masses larger than 40 kDa. This phenomenon was evidenced for a number of biological extracts, as shown in Fig. 6. While generally demonstrated, no explanation was given for this phenomenon except that small proteins are easily captured due to their more rapid diffusion within the pores of the peptide beads.

On the contrary, and at different degrees, some proteins that were detected before treatment were lost in the treated sample. Presumably, and in spite of a very large diversity of peptide structures, there are some proteins that do not find a partner to form a stable complex with. By comparing the identified proteins before and after treatment it has been possible to make a summary of the situation of noncaptured – and most likely lost proteins – from different experiments. Figure 7 summarizes the situation for four cases.

When analyzing the protein discovery thanks to the library of peptides (one or two libraries associated as a sequence) several interesting data can be selectively extracted. For the urinary proteome, the number of gene products found in the control sample was 134 and the sum of proteins of two sequential peptide ligand beads chains comprised 249 nonredundant proteins [26]. This study supplemented the known list of urine proteins with 251 new proteins. Among them were proteins of larger mass than expected (more than 200 kDa), suggesting that the concentration was also operating for proteins that were presumably from cells of urinary ducts. Among the published list of novel proteins there were membrane proteins, receptor proteins, binding proteins, and cell adhesion molecules all of them probably accounted as nonbelonging to proteins having filtered through kidney glomeruli but rather collected after filtration by duct wall cell desquamation. By comparing protein lists it appeared that 30 proteins were not detectable after treatment with the peptide ligand library. This represented about 7% of total.

The treatment of human serum with the peptide ligand library allowed revealing up to 1359 or 3869 gene products, depending on how 95% confidence was estimated [27]. From the list of identified proteins there are 75 species that belong to Ig, a group that is normally missing in depletion-based sample treatment. The relatively low overlap observed in comparison with previously published protein lists from serum proteome reinforces the notion that the peptide ligand library retains proteins that are mostly complementary to what is possible to find by using conventional methods. Some unexpected proteins came out from the analysis of bead eluates, such as nuclear proteins not known as being abundant in serum. Their presence can be explained only by a possible cell lysis occurring during the preparation of serum from blood. Another interesting observation was that basic proteins were over represented after serum treatment with the beads. Overall the study demonstrated that the bead ligand technology is effective in reducing the dynamic concentration range with the possi-

Table 4. Protein Identification from various protein extracts before and after treatment with peptide ligand libraries

<table>
<thead>
<tr>
<th>Biological extract</th>
<th>Proteins identified before peptide library treatment</th>
<th>Proteins identified after treatment with peptide libraries</th>
<th>Missing proteins</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human urine</td>
<td>134</td>
<td>383</td>
<td>36</td>
<td>25</td>
</tr>
<tr>
<td>Bile fluid</td>
<td>141</td>
<td>107</td>
<td>26</td>
<td>31</td>
</tr>
<tr>
<td>Human platelets</td>
<td>197</td>
<td>411</td>
<td>24</td>
<td>29</td>
</tr>
<tr>
<td>Red cell lysate</td>
<td>636</td>
<td>543</td>
<td>57</td>
<td>35</td>
</tr>
<tr>
<td>Cerebrospinal fluid</td>
<td>1061</td>
<td>1069</td>
<td>61</td>
<td>ND</td>
</tr>
<tr>
<td>Chicken egg white</td>
<td>41</td>
<td>147</td>
<td>1</td>
<td>32</td>
</tr>
</tbody>
</table>

Missing proteins are species that were found in the nontreated sample and were not found after ligand library interaction.
Ordering Information

Click for: Product Page

Related Information: Literature

Online ordering and pricing information are available in the following countries: the USA, the UK, Belgium, the Netherlands, Luxembourg, Israel, Sweden, Denmark, Norway, Finland, Iceland, Germany, Austria, and Switzerland.

- To see list pricing online, simply register or log in
- To order online and to see account-specific pricing, you must register with a Bio-Rad account number. If you don’t have an existing Bio-Rad account, contact your local office.

Customers outside of these countries, please contact your local Bio-Rad office for pricing information.

<table>
<thead>
<tr>
<th>Catalog #</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>163-3006</td>
<td>ProteoMiner Protein Enrichment Small-Capacity Kit, for processing 10 mg of sample, 10 prep, includes 10 spin columns, wash buffer, elution reagents, collection tubes. Yields ~170 µg protein</td>
</tr>
<tr>
<td>163-3007</td>
<td>ProteoMiner Protein Enrichment Large-Capacity Kit, for processing 50 mg of sample, 10 prep, includes 10 spin columns, wash buffer, elution reagents, collection tubes. Yields ~1 mg protein</td>
</tr>
<tr>
<td>163-3008</td>
<td>ProteoMiner Protein Enrichment Introductory Small-Capacity Kit, for processing 10 mg of sample, 2 prep, include includes 2 spin columns, wash buffer, elution reagents, collection tubes. Yields ~170 µg protein</td>
</tr>
<tr>
<td>163-3009</td>
<td>ProteoMiner Protein Enrichment Introductory Large-Capacity Kit, for processing 50 mg of sample, 2 prep, includes 2 spin columns, wash buffer, elution reagents, collection tubes. Yields ~1 mg protein</td>
</tr>
</tbody>
</table>

ProteoMiner Sequential Elution Kits

<table>
<thead>
<tr>
<th>Catalog #</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>163-3010</td>
<td>ProteoMiner Sequential Elution Small-Capacity Kit, for processing 10 mg of sample, 10 prep, includes 10 spin columns, wash buffer, sequential elution reagents, collection tubes. Yields ~180 µg protein in total, divided across 4 fractions</td>
</tr>
<tr>
<td>163-3011</td>
<td>ProteoMiner Sequential Elution Large-Capacity Kit, for processing 50 mg of sample, 10 prep, includes 10 spin columns, wash buffer, sequential elution reagents, collection tubes. Yields ~770 µg protein in total, divided across 4 fractions</td>
</tr>
</tbody>
</table>

Trademarks • Site Terms • EU Recycle Program • Privacy • Feedback

Copyright © 2009 Bio-Rad Laboratories, Inc. All rights reserved.
Ultrasensitive Detection of Trace Protein by Western Blot Based on POLY-Quantum Dot Probes

Wei Chen, Dinghua Xu, Lijiang Liu, Chifeng Peng, Yingxue Zhu, Wei Ma, Al Bian, Zhe Li, Yuanyuan Zheng, Shufang Zhu, Chuanli Xu,* and Libing Wang*

School of Food Science and Technology, State Key Lab of Food Science and Technology, Jiangnan University, Wuxi 214122, Jiangsu Province, China

In this study, we describe an ultrasensitive quantum dots (QDs)-based Western blot. With the high affinity of avidin-functionalized POLY-QDs and simplification of the detection process, this enabled the quantitative analysis of protein by Western blotting. To prepare the POLY-QDs, CdTe quantum dots were first coated with biotinylated denatured bovine serum albumin and then, via the effect of the biotin–avidin system, the biotinylated denatured bovine serum albumin-coated QDs, which had strong fluorescence, were linked together. With this series of modifications, the fluorescence intensity of CdTe-QDs was significantly increased. Using the POLY-QDs as labels, the signal of Western blotting was more sensitive in tracing the protein than traditional dyeing. In the present study, trace protein A was applied to POLY-QDs-based Western blotting as a model. The linearity of this method was from 30 pg to 1.5 mg, and the sensitivity was up to low femtomolar levels. The final fluorescence signal on the polystyrene membrane was retained for at least 40 min. The results of this study indicate that the POLY-QDs-based Western blot is an excellent quantitative analytical method for trace protein analysis.

Semi-conductor nanoparticle quantum dot (QDs) schemes have been applied widely in the biochemical field, not only because of their size-dependent tunable photoluminescence (PL) with broad excitation spectra and narrow emission bandwidths but also due to convenience as they can be coupled to biochemical molecules. Owing to the surface properties of QDs, it is possible to use a similar approach to conjugate QDs of any color to biomolecules of interest. These unique properties of QDs make them appealing as in vivo and in vitro fluorophores in a variety of biological investigations. Many researchers have used a number of methods to acquire coupling products, such as anti-quantum dot complex and antibody–quantum dot complex which have been used in immunosensors or as immunofluorescent probes. However, there are still many unsolved problems related to QDs as fluorescence probes, such as the stability of multifunctionalized QDs in different reaction systems, which obstruct the further use of QDs as fluorescence probes. Thus, many surface modifications of QDs have been studied by researchers. For example, QDs synthesized in Se/Gel-derived composite silica spheres were coated with calix[6]arene as luminescent probes for pesticides. QDs capped with 8-quinolinol were used in cell imaging, and QDs capped with 5-nitro-S,2,3-pyrazidinodiol achieved higher yield and better stability. Therefore, when CdTe-QDs were coated with DBSA, the chemical stability and photoluminescence of the QDs significantly improved. In our study, we coated the CdTe QDs with biotinylated denatured bovine serum albumin (BSA) and then prepared the conjugation of the avidin and biotin-modified denatured BSA-coated QDs (POLY-QDs). In this way, based on the POLY-QDs, we not only solved the common aggregation problem during the cross-linking reaction but also increased the stability and the photoluminescence intensity of the QDs. We then used the POLY-QDs system in Western blotting for the detection of trace amounts of protein.

Western blotting is a common method for protein analysis. The selection of secondary doxylcyclin sulfo-polyacrylamide


Published on October 3, 2006 at http://dx.doi.org/10.1021/ac060983w/292883w
RESULTS AND DISCUSSION

In this study, a new style of fluorescence probes, POLY-QDs, were applied to Western blot analysis to achieve an ultraselective detection limit. The POLY-QDs we prepared showed superior optical properties and good stability. The results and discussion on the identification of POLY-QDs and the Western blot analysis of protein A are as follows:

Preparation of POLY-QDs. In previous studies, researchers modified the QDs directly with biotin or avidin20,21 or modified the QDs indirectly by means of bovine or pig.22,23 In the present study, we carried out a new method to biotylate the QDs indirectly by coupling with biotinylated dsBA and then producing POLY-QDs via Scheme II.

Before BSA was biotinylated with NHS–biotin, it was first denatured by NaOH. Thus, the BSA was denatured to peptide chains, which have less steric hindrance than the native BSA. In addition, the disulfide bonds of BSA were opened, which makes it possible for the native capping agent TGA on the surface of QDs to be substituted by the third group of dsBA through ligand exchange.24 As a result of the above reaction, the QDs were biotinylated indirectly but more efficiently than the direct method. Then, the QDs were functionalized with avidin using a reasonable molar ratio. When the molar ratio reached 3:1, the QDs began to aggregate at the bottom of the PE tube, due to the formation of POLY-QDs. We chose a molar ratio 4:1 to ensure sufficient reaction (Figure S5 in the Supporting Information), and excess avidin was removed by centrifugation. Then, we redissolved the POLY-QDs aggregates with the same buffer solution to the certain concentration for the following research (Figure S4 in the Supporting Information). Finally, owing to the protective effect of B-BSA and avidin on the surface of the QDs,25 the stability and fluorescence intensity of QDs significantly increased (Figure S5 in the Supporting Information), which contributed to the quantitative analysis of proteins by Western blotting.

Western Blot Analysis of Protein. The critical issue in the application of QDs as labels in such immunosassays as Western blotting is the stability of QDs. Because B-BSA and avidin passivate the surface of QDs significantly, the stability of POLY-QDs was significantly increased. In addition, due to the effect of the biotin–avidin system, the optical signal of POLY-QDs was visibly amplified. Thus, POLY-QDs were very suitable for the analysis of trace multidomain protein samples using Western blotting. Through the Western blot immune reaction, the quantity of proteins was expressed by the value of the integral optical density of POLY-QDs.

A standard curve for absolute quantity of the protein A samples vs the integral optical density (IOD) was established (Figure 1). The absolute quantity of protein A samples were 1.5, 0.7, 0.3, 0.15, 0.07, and 0.03 ng. We recommend that the absolute quantity of protein samples analyzed should be less than 10 ng, otherwise the fluorescence signal will reach a saturated state, and there would be no distinct differences in the overloaded protein samples. Meanwhile, the detection results of the developed method based on the POLY-QDs were also compared with that of the commercial detection kit at the same detection conditions. At the same absolute quantity of the protein A, the detection based on the commercial kit could only show the signal of the bands until the 150 pg although the ideal limit of detection of the commercial kit could achieve 20 pg. (The detailed procedure and results are in the Supporting Information.)

Software Tool for Researching Annotations of Proteins: Open-Source Protein Annotation Software with Data Visualization

Vivek N. Bhatia, David H. Perlman, Catherine E. Costello, and Mark E. McComb*

Cardiovascular Proteomics Center, Boston University School of Medicine, 670 Albany Street, Room 804, Boston, Massachusetts 02118

In order that biological meaning may be derived and testable hypotheses may be built from proteomics experiments, assignments of proteins identified by mass spectrometry or other techniques must be supplemented with additional notation, such as information on known protein functions, protein–protein interactions, or biological pathway associations. Collecting, organizing, and interpreting this data often requires the input of experts in the biological field of study, in addition to the time-consuming search for and compilation of information from online protein databases. Furthermore, visualizing this bulk of information can be challenging due to the limited availability of easy-to-use and freely available tools for this process. In response to these constraints, we have undertaken the design of software to automate annotation and visualization of proteomics data in order to accelerate the pace of research. Here we present the Software Tool for Researching Annotations of Proteins (STRAp), a user-friendly, open-source C# application. STRAP automatically obtains gene ontology (GO) terms associated with proteins in a proteomics results ID list using the freely accessible UniProtKB and EBI GO databases. Summarized in an easy-to-navigate tabular format, STRAP results include meta-information on the protein in addition to complementary GO terminology. Additionally, this information can be edited by the user so that in-house expertise on particular proteins may be integrated into the larger data set. STRAP provides a sortable tabular view for all terms, as well as graphical representations of GO-term association data in pie charts (biological process, cellular component, and molecular function) and bar charts (cross comparison of sample sets) to aid in the interpretation of large data sets and differential analyses. Furthermore, proteins of interest may be exported as a unique FASTA-formatted file to allow for customizable re-searching of mass spectrometry data, and gene names corresponding to the proteins in the lists may be encoded in the Gadgets microformat for further characterization, including pathway analysis. STRAP, a tutorial, and the C# source code are freely available from http://cptools.sourceforge.net.
Some highlights from PBA-2009 USA (Orlando)
(Pharmaceutical and Biomedical Analysis)

Peptide therapeutics in plasma
SRM (MRM), Triple Quad MS, 5 pg/ml
D.LaineE. Chambers et al. (Waters Corp), USA

Metabolites in safety testing in drug development
Orbitrap, background subtraction, requiring >15000 mass resolution and < 5ppm mass accuracy
S.K. Showdhury (Schering Plough R.I.), USA

Metabolomics - biofluids and tissue
over 23000 metabolites (open platform): 2500 endogenous compounds, 4500 exogenous compounds, 8000 lipids, 8000 diff. peptides
MS-imaging (Anal. chem. 2009) - NIMS: 400 molecules det. with laser on teflon surface (MALDI type)
Gary Siuzdak (Scripps Res.inst.), USA

Processing of whole blood samples
Heat shock treatment (steel capillary, 16 sec, 75 deg C) disintegrated the blood cells in ca 1 mm particles, coupled to RAM SPE (6 nm pores, < 15 kD)
K.J. Siegfried Boos (Univ. Munich, Germany)

Proteomics - clinical samples
Top-down proteomics up to 40 kD
Orbitrap-ETD + triple Quad for quantitation
Elucidator soft ware, differential MS, t-tests for each peptide
R. Hendrickson (Merck Res.Lab.), USA

Structure characterization of protein drug targets
H-D exchange with deuterated water
14.6 tesla FT-ICR MS, wants 21 tesla instrument (magnet equiv. to 900 MHz NMR)
Alan G. Marshall (Florida State Univ.)

Molecular profiling and imaging by MALDI MS
Proteins in cancer tissue, also in 5000 year old mammoth (not in dinosaurs)
10μm thick slices, laser ablation gives molecular profiles from discrete areas of tissue, suggests combination with ion-mobility separation
Richard M. Caprioli (Vanderbilt Univ.School of Medicine), USA