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Human proteome analysis by using reversed phase monolithic silica capillary columns with enhanced sensitivity

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ABSTRACT

We have developed one-dimensional liquid chromatography–tandem mass spectrometry systems with meter-scale reversed phase monolithic silica-C_{18} capillary columns for human proteome analysis. When tryptic peptides from 4 μg HeLa cell lysate proteins were directly injected onto a 4-m, 100 μm i.d. monolithic silica-C_{18} column and an 8-h gradient was applied at 500 nL/min, 41,319 non-redundant tryptic peptides from 5,970 proteins were successfully identified from quadruplicate measurements; this is the best result yet reported without the use of exhaustive pre-fractionation. Because separation efficiency in the 4-m long monolithic column system (8-h gradient, 26,805 peptides identified on average) was much higher than that in a 15-cm long, conventional particle-packed column system (65-min gradient, 10,183 peptides identified), ion suppression caused by co-elution of peptides was drastically reduced, resulting in a 5-fold improvement in MS responses on average. However, we did not observe dynamic range extension for the identified human peptides, whereas 78-fold extension was observed in our previous analysis of the *Escherichia coli* proteome (Anal. Chem., 82 (2010) 2616). This was probably because the current analytical technologies are still not adequate to allow acquisition of MS/MS spectra for detected precursor ions from highly complex human peptide mixtures, even though MS sensitivity was enhanced by the improved separation in this LC system. More efficient LC separation and faster MS/MS scanning are still needed for complete human proteome analysis.

1. Introduction

The use of mass spectrometer (MS)-based shotgun-proteomic analytical systems coupled with capillary liquid chromatography (LC) has been effective in many fields of biological research [1,2]. Although LC–MS/MS analyses allow identification of thousands of proteins, it is still difficult to capture the entire proteome of interest because of the extremely high complexity and the wide dynamic range of the digested peptides obtained from proteome samples.

In order to reduce the sample complexity and the dynamic range, various pre-fractionation or multidimensional separation techniques have been reported [3–5]. These techniques have been applied to generate large-scale proteome maps for various organisms. For instance, 70 LC–MS/MS runs were required to identify 81% of the *Mycoplasma pneumoniae* proteome, which contains only 689 protein-coding genes. More complex species, *S. cerevisiae*, *D. melanogaster* and *C. elegans*, required 523, ~1,600 and 1,300 LC–MS/MS runs, respectively, to achieve 67%, 63% and 54% coverage of the protein-coding genes [6]. However, this approach might not be routinely applicable if we consider the fact that the cost of operating a high-end mass spectrometer with a capacity of 10–15 LC–MS/MS runs per day is in the range of several hundred to one thousand dollars per day. For more complex and the most important human proteome, the current proteomics technologies are not versatile enough to complete the analysis. In 2008, Hubner et al. [7] reported a more practical example, employing the isoelectric focusing (IEF) approach to fractionate 150 μg of HeLa digest peptides into 24 fractions prior to LC–MS measurement (24 runs in 2 days); this resulted in the identification of 29,265 non-redundant peptides (4,313 proteins). In 2009, Wisniewski et al. [8] combined a filter-aided sample preparation protocol with IEF pre-fractionation to identify 40,582 peptides (6,124 proteins) in 2 days LC–MS measurement of 50 μg of HeLa digest peptides. The proteome coverage obtained in these studies was still far from sufficient for complete identification of the expressed human proteome, but, given that LC–MS analysis with multidimensional separation is essential to increase the proteome coverage, LC–MS/MS measurement of a single sample within 2 days is reasonably practical.

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Alternatively, highly efficient one-dimensional LC–MS systems have been used for proteome analysis. So far, in order to obtain higher efficiency in LC separation with particle-packed columns, columns of up to 2 m long or columns packed with sub-2 μm particles have been used for analyzing various proteome samples [9–15]. The extraordinary peak capacity of 1,500 was achieved by using a 2-m, 50 μm i.d., 3 μm particle-packed column with a 2,000-min gradient at the back pressure of 140 MPa [12]. On the other hand, two types of monolithic columns such as polymer-based and silica-based columns have been developed for highly efficient separation. Polymer monolithic columns can be used for wider pH range than silica monolithic column, though commercial polymer monolith columns showed limited separation efficiency [16]. Monolithic silica materials with small silica skeletons and relatively large through-pores can achieve similar separation efficiencies to conventional particle-packed columns [17,18]. Two and more capillaries coupled monolith silica columns have been sometimes used to increase the separation efficiency [19]. Miyamoto et al. reported that a 100 μm i.d., 11.4-m monolithic silica column consisting of four capillaries achieved more than one million theoretical plates at 35.4 MPa [20]. We recently reported that triplicate LC–MS/MS analyses allowed comprehensive identification of the *Escherichia coli* proteome on a microarray scale, using a 3.5-m monolithic silica-C_{18} column at a back pressure of less than 20 MPa [21]. We also demonstrated that the proteome coverage was dramatically increased by the reduction of the ion suppression of peptides, owing to the highly efficient LC separation. Although this 'one-shot' proteomics approach is useful for the analysis of relatively less complex bacterial proteomes, it remains challenging to apply it to the more complex human proteome. Recently, Michalski et al. [22] reported that 100,000 peptides from HeLa cells were actually detected with a conventional 3 μm particle-packed column, though only about 16% of them were targeted for MS/MS, even when the most advanced mass spectrometer available was used. They clearly showed that the main factor in the failure to identify the detected peptides was the presence of co-eluted peptides in the MS/MS selection window, suggesting that improved LC separation prior to MS/MS would be effective to reduce this current limitation.

In this study, we applied our 'one-shot' approach with meter-scale monolithic silica capillary columns to analyze the human HeLa cell proteome, in order to investigate how the improvement in LC separation is related to the identification efficiency of peptides from the human proteome.

2. Experimental

2.1. Reagents and chemicals

Cytochrome C was obtained from Sigma–Aldrich (St. Louis, MO, USA). Modified trypsin was from Promega (Madison, WI, USA). Empore C_{18} disc membranes were from 3M (St. Paul, MN, USA). Water was purified by a Millipore Direct-Q system (Bedfold, MA, USA). MS-grade Lys-C and all other chemicals were purchased from Wako (Osaka, Japan). Acclaim PepMap column (3 μm particle sizes, 100 Å pore sizes, 75 μm i.d., 15 cm long) and Acclaim PepMap RSLC column (2 μm particle sizes, 100 Å pore sizes, 75 μm i.d., 15 cm long) were purchased from Dionex (Germering, Germany). ReproSil-Pur C_{18}-AQ (3 μm particle size, 120 Å pore size, 100 μm i.d., 15 cm long, Dr. Maisch, Ammerbuch, Germany) was packed in-house as previously described [23,24]. Prototype monolithic silica columns 2 m long (100 μm i.d.) were prepared by GL Sciences (Tokyo, Japan) according to the protocol previously reported [20]. Briefly, a mixture of tetramethoxysilane and methyltrimethoxysilane (9:2, v/v) was used as the silica source, and surface modification of the column was performed with octadecyldimethyl-*N*,*N*-diethyl aminosilane (ODS-DEA) (20%, v/v in dry toluene). Endcapping of silanol groups was performed with *n*-(trimethylsilyl)imidazole (20%, v/v in dry toluene) [17,25,26].

2.2. Preparation of cytochrome C digest samples

Cytochrome C (240 μg) was dissolved in 100 μL of 50 mM ammonium bicarbonate buffer containing 8 M urea. After reduction with 2 μL of 0.1 M dithiothreitol (DTT) and alkylation with 2 μL of 1 M iodoacetamide (IAA), the sample was digested with Lys-C at 37 °C for 3 h, followed by 4-fold dilution and trypsin digestion at 37 °C for O/N (enzyme-to-protein ratio of 1:100 (w/w)). The resultant sample was desalted using reversed-phase StageTips with C_{18} disk membranes [27–29].

2.3. Preparation of tryptic peptides from HeLa cells

5×10^6 HeLa cells were prepared as described previously [30]. The cell pellet was dissolved in 1 mL of 100 mM Tris buffer (pH 8.0) and protease inhibitors (Sigma) were added. After homogenization with a Dounce homogenizer (10 strokes), the resultant solution was centrifuged at 1500 × *g* for 10 min. The supernatant was processed as described above.

2.4. NanoLC-UV system

A GL Sciences MU701 capillary UV detector with a 2 nL flow cell, a Dionex Ultimate 3000 pump with FLM-3000 flow manager, and an HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland) were employed for nanoLC-UV analyses. The chromatographic data was acquired and processed by an Agilent ChemStation B.04.02 SP1 (Santa Clara, CA, USA). The injection volume was 2.5 μL. For measurement under the isocratic condition, the autosampler was inserted between the LC pump and the splitter, and the injection volume was set to be 2.5 nL after splitting the flow to the column. The mobile phase consisted of 80% acetonitrile. For gradient elution analyses, the mobile phases consisted of (A) 0.5% acetic acid (B) 0.5% acetic acid and 80% acetonitrile. A two-step linear gradient of 4–55% B with a variable time setting, 55–100% B in 1 min, and 100% B for 5 min was employed.

2.5. NanoLC–MS system

An AB SCIEX TripleTOF 5600 System (Foster City, CA, USA) equipped with a Dionex UltiMate 3000 RSLCnano pump and an HTC-PAL autosampler was employed for nanoLC–MS/MS measurement. A spray voltage of 2300 V was applied. The MS scan range was *m*/*z* 300–1500. The top 10 precursor ions were selected in each MS scan for subsequent MS/MS scans. MS scans were performed for 0.25 s, and subsequently 10 MS/MS scans were performed for 0.1 s each. To minimize repeated scanning, previously scanned ions were excluded for 12 s. The CID energy was automatically adjusted by the rolling CID function of Analyst TF 1.5. The coiled monolithic capillary columns were connected to a PicoTip emitter (20 μm i.d., 10 μm tip, New Objective, Woburn, MA, USA) with a conductive distal coating end, at which the spray voltage was applied, whereas ReproSil-packed columns with tapered ends were used as previously described [23]. The injection volume was 5 μL, and the flow rate was 500 nL/min. The mobile phases consisted of (A) 0.5% acetic acid and (B) 0.5% acetic acid and 80% acetonitrile. A three-step linear gradient of 5–10% B in 5 min, 10–40% B in 60 min, 40–100% B in 5 min, and 100% B for 10 min was employed for ReproSil-packed columns. Two 200 cm long monolithic silica columns were connected with a PicoClear (New Objective) union. A two-step linear gradient of 5–40% B in 240 min and 480 min, 40–100% B in 5 min,

Table 1
Chromatographic properties of various columns.

Column name	Column type	Diameter length	Isocratic condition				Gradient condition[d]			
			P (MPa)	K^a ($\times 10^{-14}$ m^2)	N^b ($\times 10^3$)	$N_{KPL}{}^{b,c}$ ($\times 10^3$)	t_G (min)	P (MPa)	PC	$PC_{KPL}{}^c$
Monolith Lot1	Silica monolith	100 μm, 2 m	10	9.2	171	597	400	16	325	480
Monolith Lot2	Silica monolith	100 μm, 2 m	9	10.2	187	726	400	15	314	479
ReproSil	3 μm particle	100 μm, 15 cm	7	1.0	9.38	46.9	30	12	105	179
PepMap-RSLC	2 μm particle	75 μm, 15 cm	19	0.4	5.06	9.32	30	28	117	131
PepMap	3 μm particle	75 μm, 15 cm	6	1.2	6.40	37.3	30	11	84	149

[a] The eluent viscosity = 0.00046 Pa s.
[b] N values were measured for hexylbenzene at flow rate of 1 mm/s with 80% acetonitrile.
[c] Kinetic performance limit pressure = 35 MPa.
[d] Cytochrome C digest peptides (5 pmol) were analyzed at flow rate of 1 mm/s and t_G/t_0 of 8.8. Peak capacity (PC) was calculated using 4σ estimated from $W_{1/2}$.

and 100% B for 10 min was employed for the analyses using 2-m and 4-m monolithic silica columns, respectively.

2.6. Data analysis and bioinformatics

The raw data files were analyzed by AB SCIEX MS Data Converter to create peak lists on the basis of the recorded fragmentation spectra. Peptides and proteins were identified by Mascot v2.3 (Matrix Science, London, U.K.) against UniProt/Swiss-Prot release 2011_04 (05-Apr-2011) and IPI human database (23-Mar-2011) with a precursor mass tolerance of 50 ppm, a fragment ion mass tolerance of 0.1 Da and strict trypsin specificity [31] allowing for up to 2 missed cleavages. Carbamidomethylation of cysteine was set as a fixed modification, and methionine oxidation was allowed as a variable modification. Peptides were firstly rejected if the Mascot score was below the 95% confidence limit based on the "identity" score of each peptide and their length was less than 7 amino acid residues. For protein identification, at least two confidently identified peptides per protein were required. In addition, single peptides with higher confidence ($p < 0.01$) were allowed for protein identification. False discovery rates (FDR) were estimated by searching against a randomized decoy database created by the Mascot Perl program supplied by Matrix Science.

3. Results and discussion

First, the chromatographic properties of the monolithic silica columns, as well as commercial and in-house-prepared particle-packed columns were examined by using the LC-UV system (Table 1). Two-meter monolithic silica columns provided more than 170,000 theoretical plates (N) for hexylbenzene at the back pressure of less than 20 MPa, whereas less than 10,000 theoretical plates were obtained for particle-packed columns at 11–28 MPa. Higher peak capacity (PC) values in these monolithic columns were also obtained for tryptic peptides under the gradient conditions. Note that this 5 μL direct injection system for gradient separation did not provide any peak deterioration, compared to a commercial precolumn injection system (data not shown). In order to compare the column performance with different length at different conditions, we employed the kinetic plot method [32,33]. The experimentally obtained N and PC values were extrapolated to N and PC at kinetic performance limit (N_{KPL} and PC_{KPL}), respectively, at 35 MPa, which is the upper limit of the employed LC system, according to the free-length kinetic plot. Based on these results, monolithic silica columns could provide approximately 20-fold and 3-fold higher values for N_{KPL} and PC_{KPL}, respectively, than 3 μm particle-packed columns at the flowrate of 1 mm/s and t_G/t_0 of 8.8, which are within the range used in most of proteomics applications. On the other hand, less sensitivity was obtained in the monolithic silica column systems owing to the peak dilution effect caused by the long gradient times. Note that the 2 μm particle-packed column did not provide superior results for N to the 3 μm particle-packed columns, whereas slightly higher PC was obtained at more than 2.5-fold higher back pressure. Considering PC_{KPL}, LC with 2 μm or even smaller particle-packed columns would not be suitable for gradient separation of peptides, although we did not examine other packing materials. Wang et al. [34] also reported that the use of a longer column with larger particle packed column (5 μm pellicular particles) provided the highest PC at 32 MPa. Considering the higher permeability of the monolithic column, longer monolithic silica columns with shallower gradients would be better for highly complex peptide samples, such as proteomics samples, although a longer measurement time is required.

These monolithic silica columns were subsequently used in LC–MS/MS systems for the analysis of highly complex human proteome samples. We also employed the in-house-prepared 3 μm ReproSil-packed column (15 cm × 100 μm i.d.) as a reference conventional proteomic LC–MS system. Human HeLa cell lines were selected as a model of the human proteome because HeLa cells are among the most frequently used cell samples in the field of proteomics. Table 2 lists the results. One-shot analyses with the ReproSil-packed column for 0.5 μg of HeLa digest peptides resulted in identification of 8,400 unique peptides (1,390 proteins) on average with high repeatability (relative standard

Table 2
Results of HeLa proteome analysis using various columns.

Column name	Gradient time (min)	Injected sample (μg)	Number of uniquely identified peptides	Number of uniquely identified proteins	
				IPI	UniProt
ReproSil (15 cm)[a]	65	0.5	8,400 ± 534	1,350 ± 78	1,390 ± 80
		4	10,183	1,683	1,675
Monolith-Lot 1 (2 m)	240	4	20,881	3,051	2,927
Monolith-Lot 2 (2 m)	240	4	21,064	2,980	2,839
Monolith connected[b]	480	4	26,805 ± 2,068	3,734 ± 150	3,621 ± 59
Merged[c]			41,319	5,970	4,634

[a] Repeated analyses ($n = 4$) were carried out. The average and SD values are listed.
[b] Monolith-Lot 1 and Lot 2 were connected to make a 4-m column. Repeated analyses ($n = 4$) were carried out. The average and SD values are listed.
[c] Results from the quadruplicate analyses using the connected monolith column were merged.

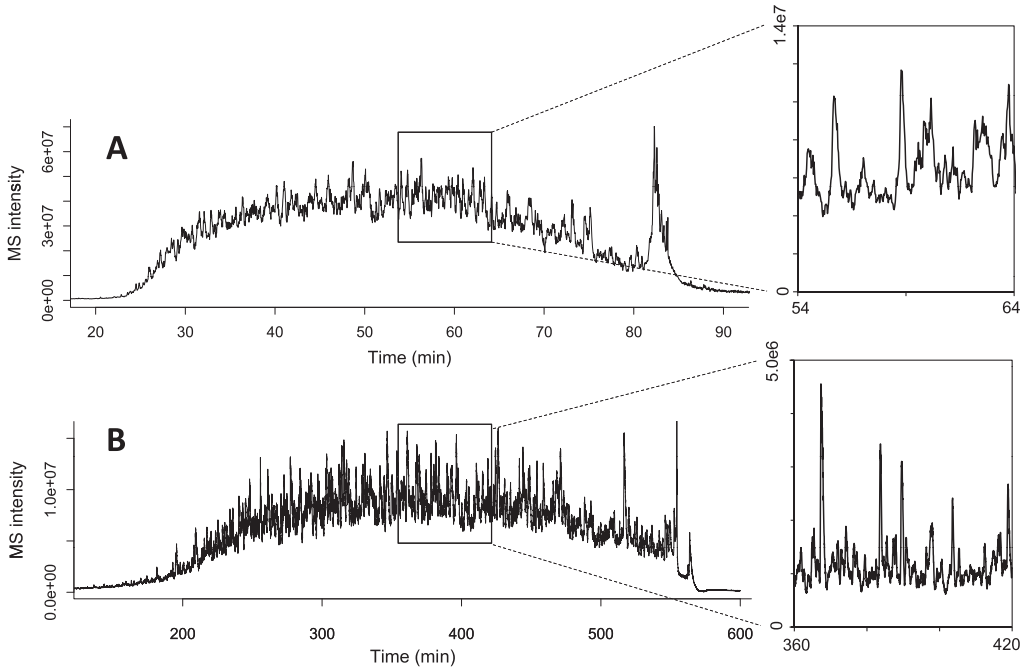

Fig. 1. Total ion current chromatograms for the analysis of HeLa digest peptides using the 15-cm ReproSil-packed column (A) and the 4-m monolithic silica column (B) with zoomed extracted ion chromatograms (m/z 700–800). Tryptic peptides of 4 μg of HeLa digest peptides were loaded onto each column, and 65-min and 480-min gradients were applied to the ReproSil-packed column and the monolithic silica column, respectively.

deviation (RSD) = 6.4% for peptides). When the injection amount was increased to 4 μg, the identification number was increased to 10,183 peptides. Two-meter monolithic silica columns with a 4-h gradient gave twice as many peptide identifications, while a smaller t_G/t_0 gradient (t_G: gradient time) was employed. As described in our previous paper [21], the peak width became broader as the injected amount was increased to 4 μg, although more peptides were identified. The variation between Lot 1 and 2 of the monolithic columns was within the repeatability in the ReproSil-packed column system in terms of the numbers of identified peptides and proteins. Further improvement was obtained when these two monolithic columns were connected to each other to make a 4-m column and an 8-h gradient was applied to the analysis of 4 μg of HeLa digest peptides. More than 26,000 unique peptides on average were identified from a single run and quadruplicate analyses resulted in the identification of more than 40,000 unique peptides. Fig. 1 shows the total ion current (TIC) chromatograms and zoomed extracted ion chromatograms (m/z 700–800) for the analysis of HeLa tryptic peptides using the ReproSil-packed column (65 min gradient) and the 4-m monolithic silica column (480 min gradient). A significantly increased number of peaks was observed in the TIC chromatogram of the monolithic silica column, though a longer measurement time was employed. The zoomed extracted ion chromatograms clearly show that higher separation efficiency was obtained in the analysis using the monolithic silica column. We examined the physicochemical properties of peptides identified uniquely by the ReproSil-packed column and the 4-m monolithic silica column. By comparing hydrophobicity as well as acidity, basicity and isoelectric points, we did not see the significant difference between two groups of peptides, indicating that the separation selectivity in each column was quite similar, as expected. Fig. 2 compares the peak areas obtained by the ReproSil-packed column and the connected monolithic silica column for the analysis of 4 μg of HeLa digest peptides. We extracted the commonly identified 3,112 peptides and plotted the peak responses. As a result, we found that the MS responses of these peptides were approximately 5-fold greater in the monolithic column system. This is presumably owing to the reduction of the ion suppression in MS due to the reduction of peptide co-elution in LC. The same phenomenon was also observed in our previous analysis of the *E. coli* proteome [21], but for the more complex human proteome analysis, a higher separation efficiency is needed to reduce the influence of ion suppression in LC–MS analysis. Actually, sensitivity enhancement was not always induced for all peptides, as shown in Fig. 3, where the dependence of sensitivity enhancement on retention times is shown. By comparing it with the TIC chromatogram in Fig. 1, we

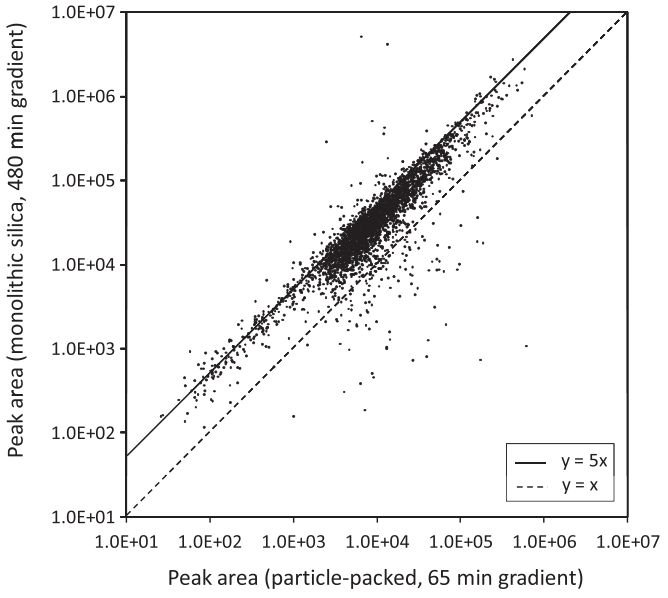

Fig. 2. Comparison of peak areas obtained by the analyses of 4 μg of HeLa digest peptides using the ReproSil-packed column with those using the monolithic silica column. Peak area values of 3,112 peptides commonly identified by the use of the particle-packed column and the monolithic silica column were plotted.

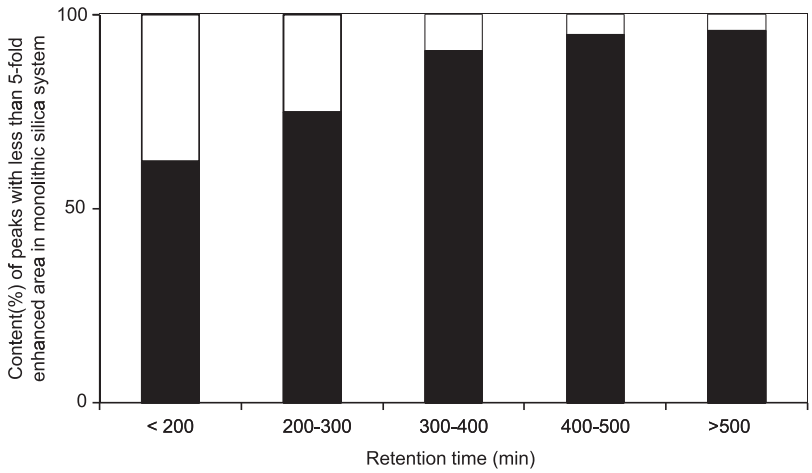

Fig. 3. Dependence of the sensitivity enhancement on the peptide retention times. The ratios of peak responses obtained from the monolithic silica column system to those of the ReproSil-packed column system were calculated. The contents of peptides with the ratios of >5 (white bar) and peptides with the ratios of ≤5 (black bar) were plotted against the peptide retention times.

found that improved separation remains necessary, especially for peptides with retention times of more than 300 min.

We also examined the effect of the LC separation on the dynamic range extension in HeLa proteome analysis, as observed in our previous analysis of the *E. coli* proteome [21]. As shown in Fig. 4, we observed a different distribution of peak responses from the monolithic silica column system for HeLa proteome analysis, whereas the distribution from the ReproSil-packed column system was similar to that in the analysis of *E. coli* proteome. The median value of the monolithic column system was almost identical to that of the ReproSil-packed column system in this study, whereas the median values in the monolithic column system were shifted to the left in the case of the *E. coli* proteome analysis. It is suggested that the current LC–MS system would not be sufficient to target the detected precursor ions for MS/MS owing to the presence of too many peptides in each survey scan, caused by the higher complexity of the HeLa digest peptides. Further improvement seems to be required in both LC and MS to improve the coverage in human proteome analysis. Considering our previous work on *E. coli* proteome where peptides would have approximately 5-times less complexity with the similar dynamic range, we can estimate that we need either 5-times more efficient LC separation, 5-fold faster MSMS scan or their combination, even though higher sensitivity was obtained in the monolithic column system by reducing the peptide co-elution.

Compared with the published HeLa cell proteome analyses using pre-fractionation approaches [7,8], we identified more peptides with a smaller amount of sample and a shorter measurement time in this study. For one-shot LC–MS analysis of HeLa proteome without pre-fractionation, we identified 1.9-fold more peptides at 60% less back pressure than those using 50 cm long, 2 μm particle packed column with the same 8-h gradient time [15], although these results cannot be directly compared, because different MS instruments with different data analysis software were used. Nevertheless, in general, the one-shot approach using monolithic silica column without pre-fractionation can minimize the required sample amount as well as the variation between samples during pre-fractionation, and therefore it is a promising approach for quantitative proteome analysis of very limited amounts of proteins from particular regions of clinical tissue samples, for instance. Very recently, Thakur et al. reported the use of a 50-cm, 75 μm i.d., sub-2 μm particle-packed column for digested peptides from human cultured cells [14]. Because of the high back pressure, they

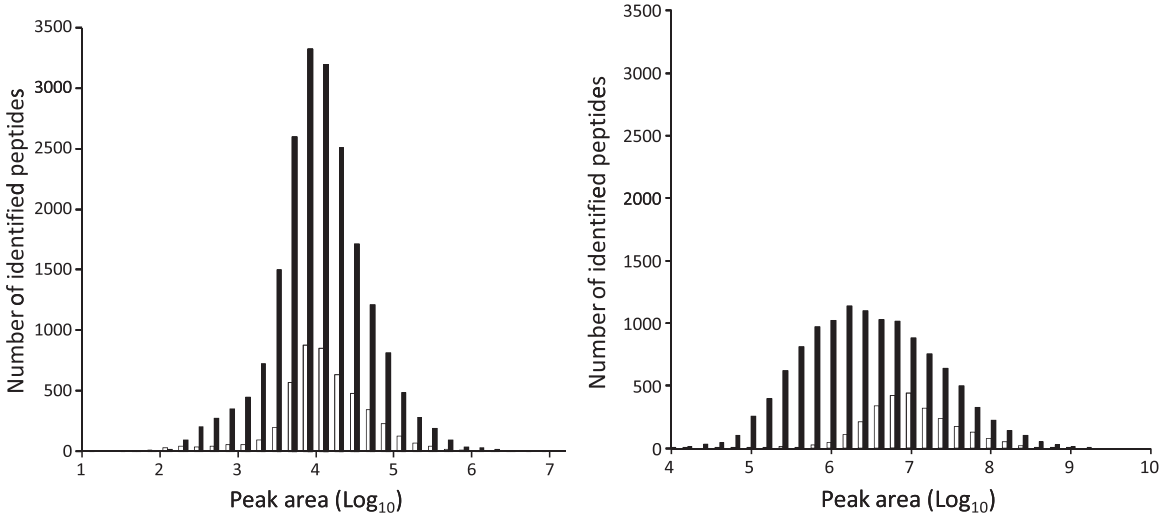

Fig. 4. Distribution of peak responses obtained from the ReproSil-packed column system (white bar) and the monolithic silica column system (black bar). The results of HeLa proteome analysis (4 μg) with the AB SCIEX TripleTOF 5600 system (A) and *E. coli* proteome analysis (4 μg) [21] with the Thermo Fisher Scientific LTQ-Orbitrap XL (B) were plotted. The bin of the *X*-axis is 0.2.

employed a flow rate of 75 nL/min, which was much less than the optimum value. However, they identified 34,877 peptides by means of 8-h gradient analysis in triplicate, which is at a similar level to our analysis. To compare our LC separation efficiency with theirs, we measured the peak width of 26,210 peptides identified by 8-h gradient analysis with the connected monolithic column. We obtained values of 51.1 s and 44.6 s for average and median peak width, respectively, whereas the corresponding values in their 50 cm, sub-2 μm particle-packed column system were 79.7 s and 64.4 s with almost the same elution window as ours (450 min). These data suggest that the comparable results from their system can be ascribed not to chromatographic improvement, but to improvement in MS detection at the lower flow rate. Thus, the combination of a long monolithic column with smaller diameter at lower flow rate should be useful to extend the proteome coverage for more complex organisms in future, although the stable operation as well as the column preparation would be difficult.

In conclusion, we applied 8-h gradient elution for analyses of HeLa digest samples on a 4-m monolithic column and were able to identify 41,319 peptides. The improved separation in this system provided higher MS sensitivity owing to the reduced ion suppression effect that is caused by co-elution of peptides within the same window. While higher MS responses were obtained in our system, dynamic range extension was not observed in this case, presumably because of the huge complexity of the samples employed. Further technology development of both LC and MS will be necessary to increase the proteome coverage. Further studies are in progress in our laboratories.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.10.059.

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