Analytica Chimica Acta xxx (2017) 1–8

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

# Integrated platform with combination of on-line protein digestion, isotope dimethyl labeling and multidimensional peptide separation for high-throughput proteome quantification

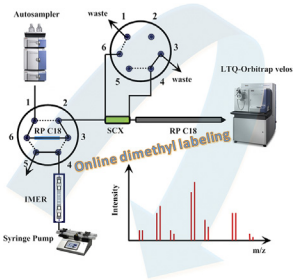
Shen Zhang [a, b, 1], Huiming Yuan [a, 1], Baofeng Zhao [a], Lihua Zhang [a, *], Yukui Zhang [a]

[a] National Chromatographic Research and Analysis Center, Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Science, Dalian, 116023, China
[b] University of Chinese Academy of Sciences, Beijing, China

## HIGHLIGHTS

- A fully integrated platform for on-line proteome quantification was established.
- Low miss-cleavage digestion with good reproducibility was achieved.
- The platform exhibited improved throughput and quantitative reproducibility.
- The integrated platform could be applied into the large-scale quantitative analysis.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Article history:*
Received 18 July 2017
Received in revised form
20 October 2017
Accepted 23 October 2017
Available online xxx

*Keywords:*
Integrated platform
Isotope dimethyl labeling
Immobilized enzyme reactor
Low miss-cleavage digestion

## ABSTRACT

In recent years, various enzymatic microreactors and on-line enzyme digestion strategies have been widely applied in high throughput proteome analysis. However, the incomplete and irreproducible digestion would introduce some unexpected variations in comparative proteome quantification when the samples are digested and then chemically isotope labeled in different aliquots. To address these problems, we developed an integrated platform for high throughput proteome quantification with combination of on-line low miss-cleavage protein digestion by an ultra-performance immobilized enzymatic reactor, on-line dimethyl labeling onto a C18 precolumn, peptide separation by two-dimensional nano liquid chromatography and MS detection. Compared to traditional off-line method, such a platform exhibits obvious advantages such as high sensitivity, throughput, accuracy, precision and ease of automation. All these results demonstrated that such a platform might become a promising technique for the quantitative proteome analysis.



* Corresponding author.
*E-mail address:* lihuazhang@dicp.ac.cn (L. Zhang).
[1] These two authors contribute equally to this work.

https://doi.org/10.1016/j.aca.2017.10.023
0003-2670/

## 1. Introduction

Stable isotope dimethyl labeling is one of the most widely used strategies for protein quantification, which globally labels the N-terminus and ε-amino group of lysine on peptides with dimethyl groups using cost-effective reagents formaldehyde and sodium cyanoborohydride [1]. Compared with other quantification methods such as SILAC [2], TMT [3] and iTRAQ [4], this method has advantages that it uses inexpensive regents and is applicable to virtually any sample with comparable or even higher labeling efficiency. Therefore, in recent years, this strategy has been widely applied into relative proteome quantification [5–8], de novo sequencing [9,10], posttranslational modification analysis [11,12], structure proteomics [13,14] and enzyme kinetics [15].

Heck and co-workers optimized this strategy and standardized three types of labeling protocols, in-solution, on-line labeling with LC-MS, and on-column using solid-phase extraction (SPE) columns [16]. Standard isotope labeling procedures usually need many manually handling steps such as sample desalting, adding labeling regents, incubation, quenching labeling, sample re-desalting, and so on, which may result in sample loss and poor reproducibility. Moreover, the manually handling procedure is time-consuming and labor-intensive. To circumvent these limitations, Raijmakers et al. established an on-line sequential isotope dimethyl labeling method, which allowed sample loading, isotope dimethyl labeling and one-dimensional (1D) LC-MS/MS analysis in a fully automated manner. Compared with off-line prefractionation, on-line multidimensional separation displays advantages such as higher sensitivity, minimal loss of sample, no vial contamination, and no sample dilution effect, shows big advantages in the analysis of limited amount of biological samples. By combining the advantageous of on-line dimethyl labeling and multidimensional separation, Wang et al. exhibited a fully automated system [17], ~1000 proteins can be quantified in ~30 h, and the proteome coverage of quantitative analysis can be improved by prolonging the multidimensional separation time. Considering on-line protein digestion might further improve the sensitivity and throughput, they tried to integrated on-line digestion into their platform but the results showed that the higher miss-cleavage rates and incomplete digestion caused by online digestion would introduce some unexpected variations in comparative quantification when the samples are online digested and then chemically isotope labeled in different aliquots [18], making the application of on-line digestion into chemical labeling based quantitative proteomics impractical. Although the influence of miss-cleavage and irreproducible digestion could be solved by prolonging the incubation time, the whole analysis throughput will be decreased and the quantitative coverage is limited because of the stopped-flow incubation [19].

To solve these problems, recently, we developed a novel ultra-performance immobilized enzyme reactor (upIMER) by immobilizing trypsin on the self-assembled graphene oxide (GO) nanosheets [20] onto polyetherimide (PEI)-modified acrylic polymer microspheres, by which high efficiency of protein digestion and $^{18}O$ labeling with low carryover can be achieved [21].

In this study, based on the upIMER, for the first time, an integrated platform for proteome quantification with combination of on-line digestion, isotope dimethyl labeling and multidimensional separation was established, by which higher sensitivity, throughput, accuracy and precision for proteome quantification was achieved compared to traditional off-line counterpart. Furthermore, such an integrated platform was further applied into the large-scale differentially quantitative proteome analysis of protein extracts from hepatocarcinoma ascites syngeneic cells with high (Hca-F) and low (Hca-P) lymph node metastasis rates. All these results demonstrated such an integrated platform will provide a promising tool for high throughput and automatic quantitative proteome analysis.

## 2. Experimental

### 2.1. Materials and reagents

Formic acid (FA), bovine serum albumin (BSA), 1,4-dithiothreitol (DTT), iodoacetamide (IAA), Trypsin (bovine pancreas), protease inhibitor cocktail, formaldehyde ($CH_2O$, 37% vol/vol), formaldehyde-d2 ($CD_2O$, 20 wt%, 98% D), sodium cyanoborohydride ($NaCNBH_3$), polyethylenimine (average Mw = 800, PEI) and urea were obtained from Sigma-Aldrich (St. Louis, MO). Acetonitrile was provided by Merck (Darmstadt, Germany). All the other chemicals were analytical grade, purchased from Kemiou Chemical Reagent (Tianjin, China). Deionized water used for all experiments was purified with a Milli-Q system (Millipore, Bedford, MA).

A precise syringe pump (Baoding Longer Pump Co., Baoding, China) was used to push the sample solution through the IMER. BCA protein assay kit was from Beyotime Institute of Biotechnology (Tianjin, China). Fused-silica capillaries (250 μm i.d. × 375 μm o.d.) were obtained from Sino Sumtech (Handan, China). Acrylic polymer microspheres with amino groups (10 μm, 1000 Å) were bought from Shenzhen Nanomicro Technology (Shenzhen, China). Venusil XBP C18 particles (5 μm, 120 Å) were ordered from Bonna-Agela Technologies (Tianjin, China).

### 2.2. Preparation of GO@PEI@polymer particles

Acrylic polymer particles with amino groups (150 mg, 10 μ, 1000 Å) were suspended in phosphate buffer (PB, pH 8.0) containing 5% glutaraldehyde (v/v) and vortexed for 3 h at room temperature. After centrifugation, the modified particles were obtained, and the unreacted glutaraldehyde was removed by flushing the particles with ethyl alcohol and water. Then, 5 mg/mL PEI was added to the suspension of the modified particles and reacted for 3 h at room temperature. After centrifugation, PEI-modified polymer particles were obtained, and the unreacted PEI was removed by washing the PEI-modified particles with ethyl alcohol and water. Finally, approximately 75 mg of PEI-modified particles was mixed with 1.5 mL (1 mg/mL) of GO suspension and vortexed for 6 h at room temperature. After centrifugation, the obtained GO@PEI@-polymer particles were washed with water, ethyl alcohol, and phosphate buffer.

### 2.3. Trypsin immobilization

The GO@PEI@polymer particles were packed into a 250 μm capillary tube; then, a 2 mg/mL trypsin solution containing 50 mM benzamidine was pumped into the capillary tube for 24 h at 4 °C. The obtained IMER was flushed by 50 mM ammonium bicarbonate (pH 8.0) to remove the residual trypsin. The IMER was stored at 4 °C for further use.

### 2.4. Sample preparation

*E. coli* cells were cultured in LB medium at 37 °C for 24 h. The cells were harvested, and then washed with ice-cold PBS for 3 times, finally suspended in extraction buffer composed of 8 M urea and 1% (v/v) protease inhibitor cocktail. The suspension was ultrasonicated for 100 s at 100 w and centrifuged at 25 000*g* for 40 min. Then, the supernatant was collected as the soluble fraction of the extracted *E. coli* whole cell lysate proteins.

Hepatocarcinoma ascites syngeneic cells with high (Hca-F) and low (Hca-P) lymph node metastasis rates were provided by Prof.

Please cite this article in press as: S. Zhang, et al., Integrated platform with combination of on-line protein digestion, isotope dimethyl labeling and multidimensional peptide separation for high-throughput proteome quantification, Analytica Chimica Acta (2017), https://doi.org/10.1016/j.aca.2017.10.023