analytical chemistry

Technical Note

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# Integration of Cell Lysis, Protein Extraction, and Digestion into One Step for Ultrafast Sample Preparation for Phosphoproteome Analysis

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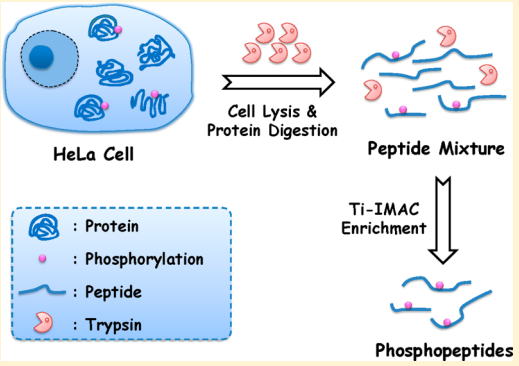
Supporting Information

**ABSTRACT:** Conventional sample preparation protocols for phosphoproteome analysis require multiple time-consuming and labor-intensive steps, including cell lysis, protein extraction, protein digestion, and phosphopeptide enrichment. In this study, we found that the presence of a large amount of trypsin in the sample did not interfere with phosphopeptide enrichment and subsequent LC-MS/MS analysis. Taking advantage of fast digestion achieved with high trypsin-to-protein ratio, we developed a novel concurrent lysis-digestion method for phosphoproteome analysis. In this method, the harvested cells were first placed in a lysis buffer containing a huge amount of trypsin. After ultrasonication, the cells were lysed and the proteins were efficiently digested into peptides within one step. Thereafter, tryptic digest was subjected to phosphopeptide enrichment, in which unphosphorylated peptides, trypsin, and other components incompatible with LC-MS/MS analysis were removed. Compared with conventional methods, better phosphoproteome coverage was achieved in this new one-step method. Because protein solubilization and cell lysis were facilitated by fast protein digestion, the complete transformation of cell pellets into the peptide mixture could be finished within 25 min, while it would take at least 16 h for conventional methods. Hence, our method, which integrated cell lysis, protein extraction, and protein digestion into one step, is rapid and convenient. It is expected to have broad applications in phosphoproteomics analysis.




Protein phosphorylation is one of the most frequently and routinely occurring post-translation modifications, and almost 30% of the eukaryotic proteome was estimated to be phosphorylated.[1] Protein phosphorylation, a reversible post-translational modification, plays a pivotal role in regulating many biologic processes such as cell division, cell growth, apoptosis, signal transduction, and so forth.[2] Aberrant phosphorylation caused by oncogenic kinase signaling is closely related to malignant tumors.[3] Therefore, global profiling of the phosphoproteome was necessary for systematically understanding the cellular behavior. In recent decades, advances in mass spectrometry have enabled itself to be a powerful technology for phosphoproteomics analysis.[4] Moreover, the development of efficient sample preparation methods, e.g., the FASP method, also largely contributes to in-depth proteomics analysis.[5] Hence, sample preparation is also a key step for phosphoproteome analysis. The conventional protocol roughly includes the following steps: (1) cell lysis; (2) protein extraction and optional protein precipitation followed by redissolution; (3) protein digestion; and (4) phosphopeptide enrichment.[6−8] These steps are always performed separately, which make sample preparation laborious and time-consuming. Therefore, it is indispensible to develop a convenient and efficient sample preparation method for phosphoproteomics analysis.

To speed up the sample preparation, many protocols emerged to optimize the relatively time-consuming part, i.e., protein digestion.[9] Many techniques, such as using high pressure, sonication, and enzyme immobilization, have been applied to accelerate protein digestion.[10−13] It is well-known that the higher trypsin-to-protein ratio, the faster is protein digestion.[14] Compared with the above approaches, digestion with a high trypsin-to-protein ratio is a straightforward approach to reduce the digestion time. Unfortunately, more trypsin autolysis peptides will produce at a high ratio. To avoid the interference of intact trypsin and its autolysis peptides with downstream LC-MS/MS analysis, a trypsin-to-protein ratio of less than 1:25 is usually recommended for proteomics analysis. However, when trypsin was immobilized onto solid beads, the trypsin autolysis was largely suppressed and the immobilized trypsin could be easily removed.[15−17] Thus, a huge amount of trypsin could be applied for fast protein digestion in proteomics analysis. It was reported that protein digestion aided by

**Received:** January 17, 2014
**Accepted:** June 23, 2014
**Published:** June 23, 2014

   dx.doi.org/10.1021/ac5002146 | *Anal. Chem.* 2014, 86, 6786−6791

immobilized trypsin can be completed within a few minutes.[18] Nonetheless, the immobilized trypsin was not widely used in routine proteomics analysis, probably owing to the inconvenient procedure and the sample loss resulting from the nonspecific adsorption of peptides by the solid phase surface.

Interference by trypsin and its autolysis fragments was a serious problem for proteomics analysis if protein digestion was conducted at a high trypsin-to-protein ratio. However, it is quite different for phosphoproteome analysis, since the phosphopeptide purification is required prior to LC-MS/MS analysis. The phosphopeptide enrichment approach had been demonstrated to have extremely high specificity. For example, phosphopeptides could be specifically isolated from a peptide mixture with more than 100-fold excess of unphosphorylated peptides by Ti (IV)-IMAC beads.[19] It was likely that excess trypsin and its autolysis fragments could be completely removed through the phosphopeptide enrichment step. Therefore, a large amount of free trypsin might be implemented to facilitate protein digestion for rapid sample preparation in phosphoproteome analysis. In addition, we reasoned that a large amount of trypsin may also accelerate the process of cell lysis and protein extraction, as small proteins especially peptides do not have much difficulty for solubilization. On the basis of this assumption, we developed a fast concurrent lysis-digestion strategy for phosphoproteome analysis. In this new method, the cell pellets are directly exposed to a large amount of trypsin for cell lysis, protein extraction, and digestion. The resulted digest is directly subjected to phosphopeptide enrichment followed by RPLC-MS/MS analysis of enriched phosphopeptides. This new protocol was applied to analyze the phosphoproteome of complex samples. A higher coverage was observed compared with conventional protocols. More importantly, it took only 25 min for the transformation of cell pellets to protein digest.

## EXPERIMENTAL PROCEDURES

**Sample Preparation with the One-Step Method.** The lysis-digestion buffer containing 10 mM DTT, 1 mM NaF, 1 mM $Na_3VO_4$, 0.8% NP-40, and 50 mM Tris-HCl buffer (pH 8.2) was prepared for concurrent lysis and digestion. To a centrifuge tube with HeLa cell pellets (~100 000 cells), 100 $\mu$L of lysis-digestion buffer and a suitable amount of trypsin were added. The tube was vortexed for several seconds to break the cell membrane and then was put into a water bath at 37 °C under ultrasonication for a period of time for cell lysis and simultaneous protein digestion. The generated peptide mixture was placed at −20 °C for later phosphopeptide enrichment.

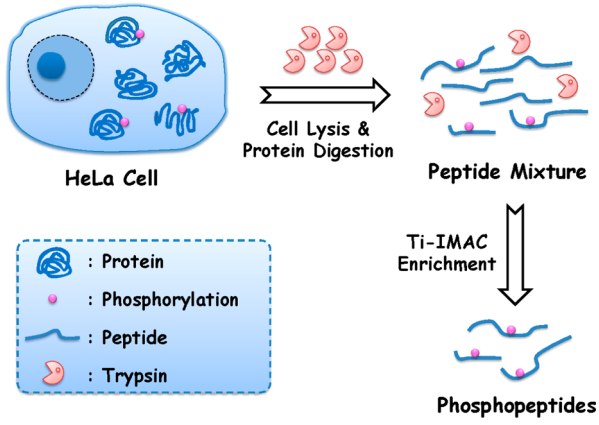
**Sample Preparation with Conventional Methods.** Two conventional sample preparation protocols were used for comparison. Conventional protocol A was the protocol often used in our lab.[20] HeLa cell pellets (~100 000 cells) were lysed in ice-cold lysis buffer containing 8 M urea, 50 mM Tris-HCl (pH 7.4), 65 mM dithiothreitol (DTT), 1 mM EDTA, 0.5 mM EGTA, phosphatase inhibitor (1 mM NaF and 1 mM $Na_3VO_4$), 1 mM PMSF, 1% Triton X-100, and 2% protease inhibitor cocktail in an ice bath and then were sonicated for 25 min. The cell lysate was centrifuged at 25 000$g$ at 4 °C for 30 min. The supernatant was added with five volumes of ice-cold precipitation solution (ethanol/acetone/acetic acid = 50:50:0.1) at −20 °C. The precipitated proteins were centrifuged at 25 000$g$ at 4 °C for 30 min and then washed stepwise by ice-cold acetone and 75% ethanol. The extracted proteins were redissolved in 50 mM Tris-HCl (pH 8.2) containing 8 M urea, and the protein concentration was determined by the Bradford assay. The reduction/alkylation step was performed by adding 20 mM DTT at 37 °C for 2 h and 40 mM IAA at room temperature in darkness for 40 min. The solution was diluted to 1 M urea by 50 mM Tris-HCl, and the proteins were digested overnight at 37 °C with an enzyme-to-protein ratio of 1/25 (w/w).

The protocol B was reported by Zhou et al.[21] The HeLa cells pellets (~100 000 cells) were lysed in 50 mM Tris-HCl buffer (pH 8.2) containing 8 M Urea, phosphatase inhibitor (1 mM NaF and 1 mM $Na_3VO_4$), and 2% protease inhibitor cocktail with ultrasonication for 25 min. Protein concentration measurement, reduction, alkylation, and digestion were performed as above. The digests were desalted using Desalting Tips, dried down in vacuum, and stored at −20 °C. The other experimental procedures were given in detail in the Supplementary Experimental Procedures, Supporting Information.

## RESULTS AND DISCUSSION

Recently, a one-stop method enabling the transformation of cells into tryptic digest in a single tube was developed for proteomics analysis.[22] The method was efficient but relatively tedious because multiple steps of cell lysis, protein denaturation, and protein digestion were performed separately in the tube. Furthermore, overnight digestion was required because protein digestion was slow where a small amount of trypsin was applied (trypsin-to-protein ratio of 1:20). Here, in this study, we reported a much faster one-step sample preparation method for phosphoproteome analysis. Three sample preparation steps, i.e., cell lysis, protein extraction, and protein digestion, were integrated into one step in one tube. As shown in Scheme 1,

**Scheme 1. Workflow for the "One-Step" Concurrent Lysis-Digestion Method for Phosphoproteome Analysis[a]**




[a]A large amount of trypsin was applied to speed up the sample preparation.

harvested cells were first suspended in a lysis buffer with the presence of a huge amount of trypsin and then sonicated in a water bath at 37 °C for a period of time. The ultrasonication would facilitate the disruption of cells and expose internal proteins to a large excess of trypsin which accelerates the digestion. Also, the initial tryptic hydrolysis made the proteins smaller, more soluble in lysis buffer, and also more easily further digested by trypsin. Therefore, proteins were simultaneously extracted and digested within one single step for this


dx.doi.org/10.1021/ac5002146 | Anal. Chem. 2014, 86, 6786−6791

concurrent lysis-digestion method. Finally, the peptide mixture was subjected to Ti (IV)-IMAC purification followed by RPLC-MS/MS analysis. The residue trypsin, the unphosphorylated peptides, other contaminants from the lysis buffer, and the cell debris were all removed in this step. Due to this enrichment step, a much large amount of trypsin could be applied to speed up protein digestion, which made this method extremely fast.

To test the feasibility of fast digestion using a large amount of free trypsin, we first investigated if excess trypsin and its autolysis product left in the sample would interfere with subsequent phosphopeptide identification. HeLa cell proteins were digested overnight at different trypsin-to-protein ratios (w/w) of 1:25, 1:1, 5:1, and 10:1, respectively. Phosphopeptides enriched from the above digests were identified by LC-MS/MS analysis. As Figure S-1, Supporting Information, shows, there was barely any difference in the numbers of identified unique phosphorylation sites with the increase of trypsin amount. Even the amount of trypsin increased to 10-fold of proteins, as many phosphorylation sites as those identified in the control group (trypsin-to-protein ratio of 1:25). Clearly, after phosphopeptide enrichment, excess trypsin and its autolysis products in the peptide mixture could be completely removed and did not compromise subsequent phosphopeptide identification. Furthermore, the enrichment specificities (i.e., the percentage of phosphopeptide identifications in all peptide identifications) of these experiments were all as high as about 95%, which confirmed that the Ti (IV)-IMAC enrichment step effectively removed the non-phosphopeptides.

The above experiment indicated that a large excess of trypsin left in the sample did not compromise the performance of phosphopeptide identification. On this basis, we further investigated if a large amount of trypsin could be applied to speed up the protein digestion in the concurrent lysis-digestion protocol. Various amounts of trypsin were added to different centrifuge tubes with the same amount of HeLa cell pellets (~100 000 cells, ~40 $\mu$g of proteins) to reach trypsin-to-protein ratios (w/w) of roughly 1:25, 1:5, 1:1, and 3:1, respectively. Each tube was sonicated for 1 h for lysis and digestion. Then, phosphopeptides were enriched for LC-MS/MS analysis. As shown in Figure 1A, the number of identified unique phosphorylation sites increased with the increase of the trypsin-to-protein ratio from 1:25 to 1:1. About 900 unique phosphorylation sites were identified at a ratio of 1:25, while almost 1600 unique phosphorylation sites could be detected at a ratio of 1:1. Clearly, the digestion with trypsin-to-protein ratios routinely used in proteomics analysis (typically less than 1:25) was not efficient and did not go to completion within 1 h. However, when the ratio increased to 1:1, the cells were efficiently lysed and digested; thus, the highest number of phosphorylation sites was identified. No more increase was observed with the further increase of trypsin amount (over ratio of 1:1). This is because protein digestion was completed within 1 h for trypsin-to-protein ratio over 1:1.

The trypsin-to-protein ratio of 1:1 was selected for the following experiments because fast cell lysis and protein digestion could be achieved. We then investigated the influence of digestion time on the phosphoproteomics analysis for this concurrent lysis-digestion protocol. The digestion times of 25 min, 45 min, 1 h, and overnight were evaluated. For the overnight experiment, the concurrent lysis and digestion process was conducted under ultrasonication for the first 1 h and then followed by incubating in a water bath overnight.

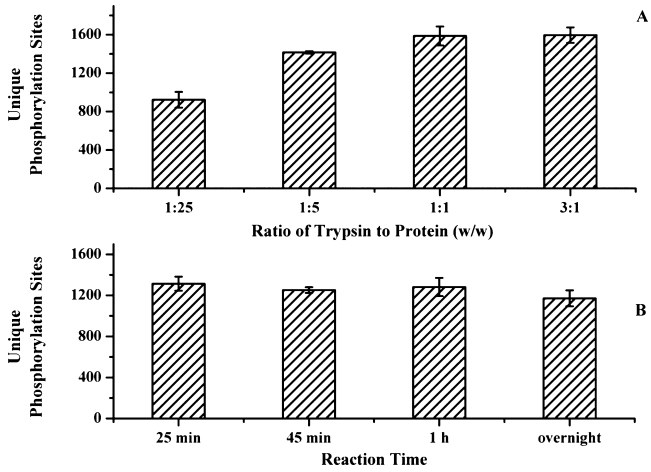



**Figure 1.** Optimization of trypsin amount and reaction time for the one-step method. (A) The numbers of unique phosphorylation sites identified with different trypsin-to-protein ratios. Digesting $10^5$ HeLa cells with different amounts of trypsin under ultrasonication for 1 h. Half of the enriched phosphopeptides was loaded per MS analysis. The numbers shown are the average for four sample preparation experiments. The error bar was the standard deviation. (B) The numbers of unique phosphorylation sites identified with different reaction times. The conditions were as above except that the trypsin-to-protein was 1:1.

Results in Figure 1B indicated that 25 min was sufficient for digestion in this new method because no more phosphorylation sites were identified with a further increase of digestion time. One can imagine that a mutual-promotion process occurred in the concurrent lysis-digestion process: the cell lysis exposed proteins to trypsin, which facilitated protein digestion and, in turn, fast protein digestion, making protein smaller and more soluble in the lysis buffer which promoted further cell lysis. Therefore, the cell pellets could be processed into protein digest for phosphoproteome analysis in only one step in one tube in very short time.

After the above investigations, a trypsin-to-protein ratio of about 1:1 and reaction time of 25 min were determined as the optimal conditions for this one-step sample preparation for phosphoproteome analysis. In order to assess its performance, this new protocol was compared with the other two conventional protocols (Protocol A and Protocol B, depicted in the Experimental Procedures section) for phosphoproteomics analysis. The cell lysis, protein extraction, and protein digestion were separately performed for Protocol A and Protocol B, both of which were tedious and labor intensive as shown in Table 1. The three protocols were applied to analyze the same cell sample ($10^5$ HeLa cells), and all the enriched phosphopeptides were loaded for LC-MS/MS analysis. For each protocol, three sample preparation experiments were performed in parallel. The three experiments using this one-step protocol led to the identification of 2987 unique phosphorylation sites, while only 2682 and 2020 unique phosphorylation sites were identified for Protocol A and B, respectively (Table 1). Compared with Protocol A and B, 11% and 48% more phosphorylation sites were identified using this new method, respectively. For the three experiments, the average numbers of identified unique phosphorylation sites and their RSD values were 1654 (RSD = 3.8%) for the one-step method, 1515 (RSD = 3.0%) for Protocol A, and 1171 (RSD = 10.0%) for Protocol B, respectively. The above data indicated


dx.doi.org/10.1021/ac5002146 | Anal. Chem. 2014, 86, 6786−6791

**Table 1. Comparison of the Time Spent in Every Step of the New Method and Two Conventional Protocols and the Cost Using Two Types of Trypsin, i.e., Sequencing Grade Trypsin and TPCK Treated Trypsin for Analyzing $10^5$ HeLa Cells**

| | group | | |
|---|---|---|---|
| time | one-step | protocol A | protocol B |
| cell lysis and protein extraction | 25 min (alkylation omitted) | ~12 h (protein precipitation included) | 25 min |
| reduction | | 1 h | 1 h |
| alkylation | | 40 min | 40 min |
| digestion | | ~16 h | ~16 h |
| enrichment | ~2 h | ~2 h | ~2 h |
| total time | ~2.5 h | >28 h | >16 h |
| unique phosphorylation sites | 2987 | 2682 | 2020 |
| cost (using TPCK treated Trypsin) | ~\$1/$10^5$ HeLa cells | ~\$1/$10^5$ HeLa cells | ~\$1/$10^5$ HeLa cells |
| cost (using sequencing grade Trypsin) | ~\$46/$10^5$ HeLa cells | ~\$1.5/$10^5$ HeLa cells | ~\$1.5/$10^5$ HeLa cells |

that this new method as well as two conventional methods could repeatedly generate a similar number of phosphorylation site identifications. Moreover, more than 45.0% of phosphorylation sites was identified within at least two replicates of sample preparation experiments for all the three methods, which further indicated the good reproducibility of three methods (Figure S-2.A, Supporting Information). Nonetheless, the overlap was still a little lower than that identification overlap for replicate LC-MS/MS analysis of the same peptide sample. This was reasonable since additional variations would be generated during the sample preparation steps in addition to the under-sampling problem during the MS detection.[23] However, the overlaps for the new method were comparable to those of Protocol A and B, indicating its reproducibility was at least as good as those conventional methods.

We compared the complementarities for the phosphorylation sites identified by the three approaches. It was found that similar overlaps, about 38%, between every two approaches were obtained (Figure S-2.B, Supporting Information). The low overlap may be partly attributed to the reason that different sample preparation methods extracted slightly different proteins. Compared with two conventional approaches, this new method identified more unique phosphorylation sites. The efficiencies for the identification of phosphorylation sites on membrane proteins for the three approaches were compared. The membrane proteins were predicted by the topology algorithm TMHMM v2.0, which was a hidden Markov model-based predictor for transmembrane helices in protein sequences.[24] It was found that the percentages of membrane proteins for this one-step method, Protocol A, and Protocol B were 4.5%, 5.8%, and 4.9%, respectively. Clearly, those approaches have similar performance in the identification of phosphorylation sites on membrane proteins.

As in conventional protocols, DDT was added to reduce the disulfide bonds on proteins, but the alkylation step was omitted in this new method. Therefore, the cysteine containing peptides might cross-link together via reformation of disulfide bonds in the downstream processes and hence cannot be identified. We compared the percentage of cysteine containing phosphopeptides among the identified phosphopeptides for the three protocols. It was revealed that the percentages were 3.8%, 11.0%, and 7.8% for the new protocol, Protocol A, and Protocol B, respectively. Obviously, this new protocol yielded the lowest number of cysteine containing phosphopeptides, indicating it was not efficient enough to identify these phosphopeptides. Considering the cysteine-containing phosphopeptides accounting for only about 10% of the total phosphopeptides, the loss of these peptides did not have notable effect on the whole coverage. However, if the cysteine containing phosphopeptides is a main concern, alkylation could be simply achieved by addition of IAA to the solution after the concurrent lysis-digestion process.

No protease inhibitor was added in the lysis-digestion buffer for the one-step method, while the inhibitors were added for cell lysis in Protocols A and B to inhibit the activities of endogenous proteases in the cells. It is interesting to investigate if seriously unspecific cleavages occurred in this new method. To investigate the protease cleavage specificities, a semitrypsin search was performed for the MS data acquired by the three methods. The tryptic digestion specificities, represented by the percentage of fully tryptic phosphopeptides among all phosphopeptides, were determined to be 86.5%, 88.8%, and 88.3% for Protocol A, Protocol B, and the one-step method, respectively. The specificities were all quite good for these protocols indicating no seriously nonspecific cleavage occurred during the sample preparation. For Protocols A and B, protease inhibitor cocktail (2%) was added to lysis buffer to inhibit the endogenous proteases during cell lysis. After cell lysis, the buffers were diluted 8-fold to reduce the concentrations of urea and inhibitors to a level that did not seriously affect the trypsin activity during digestion. While in this lysis-digestion concurrent method, no dilution was performed. Hence, adding too much inhibitor may seriously inhibit trypsin activity. Considering that the concentration of trypsin is far higher than that of any endogenous protease presented in the cells, the endogenous proteases were not likely to generate many nonspecific cleavages during the sample preparation step in the new protocol. Therefore, we did not add any protease inhibitor for this new protocol. The tryptic digestion specificity data also indicated that the addition of protease inhibitors is not necessary for this new protocol because of the presence of a huge amount of trypsin and the short digestion time.

We further investigated the recovery of phosphopeptides in the above three protocols. The peptides generated from the same cell sample ($10^5$ HeLa cells) by the one-step method, Protocol A, and Protocol B were labeled separately with heavy, intermediate, and light stable isotope dimethyl labels and then were combined and subjected to phosphopeptide enrichment. The percentages of phosphopeptides quantified with log 2 (ratio H/M) and log 2 (ratio H/L) over 1 were 79.7% and 83.9%, respectively (Figure S-3, Supporting Information). This indicated that the phosphopeptides from the one-step method had much higher recovery than that from the two control methods. Recently, it was reported that the formation of salt bridges between R/K cleavage sites and phosphoamino acids pS/pT made the digestion of these sites less efficient.[25] To improve the digestion efficiency, increasing trypsin concentration up to a trypsin-to-peptide ratio of 1:10 led to a significant gain in phosphopeptide identification. Therefore, the high recovery of phosphopeptides in this concurrent lysis-digestion approach may partly be due to more phosphopeptides being generated from the high resistant region of phosphoproteins.


dx.doi.org/10.1021/ac5002146 | Anal. Chem. 2014, 86, 6786−6791

This one-step method was further applied to analyze the phosphoproteome of another type of cancer cells, i.e., HepG-2 cells. The average numbers of identified unique phosphorylation sites for one-step method, Protocol A, and Protocol B were 1452 (RSD = 5.1%), 1167 (RSD = 0.5%), and 965 (RSD = 10.0%), respectively. The best results were obtained for the one-step method, which was consistent with the results obtained with HeLa cells. The performance of this one-step method for quantitative phosphoproteomics was also investigated. Two aliquots of HeLa cells were processed by the one-step method in parallel, and the generated peptides were labeled separately with light and heavy stable isotope dimethyl labels. After pooling the above two samples, the phosphopeptides were enriched and quantified by LC-MS/MS. For comparison, the same samples were also processed by Protocols A and B. The numbers of quantified unique phosphorylation sites were 1465, 1322, and 965 for one-step method, Protocol A, and Protocol B, respectively. The one-step method yielded the highest number of quantifications. The percentages of phosphorylation sites quantified within the log 2 ratio range of −1 to 1 were 99.0%, 98.6%, and 97.7% for the three methods, respectively (Figure S-4, Supporting Information). Thus, the accuracy for quantification was comparable to these well established methods. Clearly, this new method enabled the quantitative phosphoproteome analysis with higher sensitivity and much shorter sample preparation time.

It was noteworthy that the time of sample preparation in this new method was only 25 min, but it took many hours for the conventional sample preparation protocols (28 h for Protocol A and 16 h for Protocol B, Table 1). Obviously, the one-step method showed its superiority over traditional methods in ultrafast sample preparation for phosphoproteome analysis. The trypsin activity assay indicated that the hydrolysis rate increased dramatically with the increase of trypsin amount (Figure S-5, Supporting Information). Therefore, it can be concluded that the excellent performance of this new protocol was attributed to the accelerated enzymatic hydrolysis due to the high concentration of trypsin applied. The large amount of trypsin used in this new protocol greatly saved the sample preparation time. However, the reagent cost increased drastically if expensive sequencing grade trypsin was used. It is lucky that the performance of proteome analysis was not correlated with the price of trypsin applied.[26] It was reported that the inexpensive TPCK treated trypsin could be an alternative of the sequencing grade trypsin (1000-fold of the cost of TPCK treated trypsin) for proteomics experiments. TPCK treated trypsin was also successfully used in large phosphoproteome analysis where more than 10 000 phosphorylation sites were identified.[27] In this study, this inexpensive TPCK treated trypsin was used ($0.7 per mg). For the transformation of HeLa cell pellets (~100 000 cells) into peptides, 40 μg of trypsin was added which only cost 3 cents. Hence, this method was not expensive at all. The FASP method is an excellent sample preparation method for proteomics analysis.[5] We compared our cost with the FASP method where a small amount of trypsin was applied. If TPCK treated trypsin was used for the two methods, the cost of our method was mainly from Eppendorf GELoader tips (about $1 per tip), while that of FASP was from Centrifugal Filter Devices ($7 each), not including the cost of phosphopeptide enrichment. If sequencing grade trypsin was used, the cost of our method was mainly from sequencing grade trypsin ($45/40 μg), while FASP was still from Centrifugal Filter Devices ($7 each) because the cost of a small amount of trpsin was negligible. It should be mentioned that, if a huge amount of sample (over 10 mg proteins) needs to be processed, our new method is relatively expensive even though TPCK treated trypsin is used. This method is best suited to process a relatively small amount of sample.

## CONCLUSIONS

In this study, we developed a novel concurrent lysis-digestion method, in which the tedious three-step procedures of cell lysis, protein extraction, and protein digestion were simplified into a one step. Cell lysis facilitated protein digestion by trypsin, and in turn, the fast protein digestion promoted further cell lysis. Because the excess trypsin could be removed in the phosphopeptide enrichment step, a trypsin-to-protein ratio as high as 1:1 could be applied to speed up the lysis-digestion step. Compared with the minimum of 16 h of sample preparation in conventional methods, this new method enabled the transformation of cells into tryptic digest within only 25 min. This novel method will find broad applications in phosphoproteomics studies because of its rapid, convenient procedure and its excellent performance.

## ASSOCIATED CONTENT

### Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

This work was supported by the China State Key Basic Research Program Grant (2013CB911202, 2012CB910101, 2012CB910604), the Creative Research Group Project of NSFC (21321064), the National Natural Science Foundation of China (21275142, 21235006, 81161120540, 81361128015), Chinese National Key Project (2012ZX10002009-011), and Analytical Method Innovation Program of MOST (2012IM030900).

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