Journal of Proteomics 196 (2019) 33–41

Contents lists available at ScienceDirect

Journal of Proteomics

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

Sensitive profiling of cell surface proteome by using an optimized biotinylation method

Yanan Li[a,b], Yan Wang[a,b], Jiawei Mao[a,b], Yating Yao[a,b], Keyun Wang[a], Qinglong Qiao[a], Zheng Fang[a,b], Mingliang Ye[a,*]

[a] CAS Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences (CAS), Dalian 116023, China
[b] University of Chinese Academy of Sciences, Beijing 100049, China

ARTICLE INFO

Keywords:
Cell surface proteins
Biotinylation
Plasma membrane proteins
Proteomics
Breast cancer cell lines

ABSTRACT

Cell surface proteins are responsible for many critical functions. Systematical profiling of these proteins would provide a unique molecular fingerprint to classify cells and provide important information to guide immunotherapy. Cell surface biotinylation method is one of the effective methods for cell surface proteome profiling. However, classical workflows suffer the disadvantage of poor sensitivity. In this work, we presented an optimized protocol which enabled identification of more cell surface proteins from a smaller number of cells. When this protocol was combined with a tip based fractionation scheme, 4510 proteins, including 2055 annotated cell surface-associated proteins, were identified with only 20 microgram protein digest, showing the superior sensitivity of the approach. To enable process 10 times fewer cells, a pipet tip based protocol was developed, which led to the identification of about 600 cell surface-associated proteins. Finally, the new protocol was applied to compare the cell surface proteomes of two breast cancer cell lines, BT474 and MCF7. It was found that many cell surface-associated proteins were differentially expressed. The new protocols were demonstrated to be easy to perform, time-saving, and yielding good selectivity and high sensitivity. We expect this protocol would have broad applications in the future.

Significance: Cell surface proteins confer specific cellular functions and are easily accessible. They are often used as drug targets and potential biomarkers for prognostic or diagnostic purposes. Thus, efficient methods for profiling cell surface proteins are highly demanded. Cell surface biotinylation method is one of the effective methods for cell surface proteome profiling. However, classical workflows suffer the disadvantage of poor sensitivity. In this work, we presented an optimized protocol which enabled identification of more cell surface proteins from a smaller number of starting cells. The new protocol is easier to perform, time-saving and has less protein loss. By using a special pipet tip, sensitive and in-depth cell surface proteome analysis could be achieved. In combination with label-free quantitative MS, the new protocol can be applied to the differential analysis of the cell surface proteomes between different cell lines to find genetically- or drug-induced changes. We expect this protocol would have broad application in cell surface protein studies, including the discovery of diagnostic marker proteins and potential therapeutic targets.

1. Introduction

Cell surface proteins are responsible for communication with the environment and play important roles in many fundamental biological processes, e.g. cell-cell interactions, cell signaling via receptors and transportation of ion and solutes [1,2]. So far, > 60% of the protein-based drug targets are located on the cell surface and cell surface proteins are often used as markers for classification and isolation of specific cell types [3–5]. More recently, cell surface protein-directed monoclonal antibodies (mAbs) have been in clinical use for the treatment of cancers, autoimmune diseases and so on [2,6,7]. Thus, efficient methods for profiling cell surface proteins are highly demanded. Nevertheless, due to the heterogeneity, overall low cellular abundance, and highly hydrophobic character, cell surface proteins are underrepresented and difficult to achieve large-scale identification.

In recent years, many methods have been developed for the isolation of cell surface proteins, mainly relying on either difference of physicochemical properties, or selectively isolating via chemical labeling [8]. The two chemical labeling approaches, i.e. cell surface capturing (CSC) and cell surface biotinylation, are popular methods for

* Corresponding author.
E-mail address: mingliang@dicp.ac.cn (M. Ye).

https://doi.org/10.1016/j.jprot.2019.01.015
Received 19 September 2018; Received in revised form 18 January 2019; Accepted 22 January 2019
Available online 29 January 2019
1874-3919/

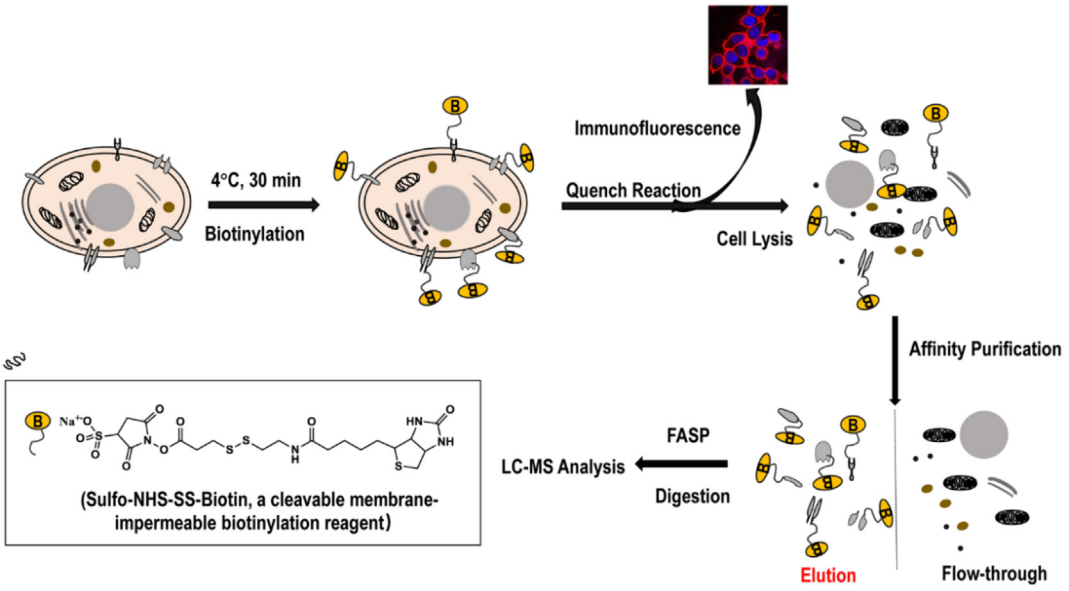


Fig. 1. Schematic diagram of the workflow for cell surface protein enrichment.

the analysis of cell surface proteins. CSC technology was first developed in 2009 by Wollscheid et al. [5]. Since the vast majority (~ 90%) of the surfaceome is predicted to be glycosylated, the method covalently labels extracellular N-glycan moieties using hydrazide chemistry on live cells, enables the analysis of the cell surface N-glycoproteomics. Thus high specificity and selectivity (~ 90%) for cell surface proteins could be achieved [9–12]. However, probably because the extensive sample-handling steps result in severe sample loss, this method requires large amounts of starting material (10^7 to 10^8 cells per experiment) [13], which may not be always available, especially for stem cells. Besides, the CSC technology identify relative small numbers of cell surface proteins (typically < 500 proteins) and cannot obtain the glycan structure information [12,14]. While cell surface biotinylation method, by selectively covalent labeling of cell surface proteins with a biotinylated reagent, can keep the glycan intact and is another popular method to isolate cell surface proteins with good selectivity. The workflow of biotinylation method is simpler and can keep the glycan structure information. Besides, cell surface biotinylation method has been applied to in vivo settings (i.e., perfusion of animal vasculatures) [15]. Nevertheless, a large number of starting cells (typically ~ 10^7 cells per experiment) was often required, and only moderate numbers of cell surface proteins and transmembrane proteins were identified (typically < 1000 cell surface proteins) [8,9,16–19].

Herein, we optimized the protocol of cell surface biotinylation method, mainly the elution procedure, and more cell surface proteins were identified from the reduced amount of starting cells (~ 10^6 cells per experiment). The new protocol is easier to perform, time-saving and has less protein loss. Finally, the new protocol was applied to the detection and quantification of cell surface proteomes for two breast cancer cell lines, BT474 and MCF7. In combination with label-free quantitative MS, a number of cell surface-associated proteins showed either up- or down-regulated in both breast cell lines, including Her2, which is known to be overexpressed in BT474 cells. We expect this protocol would have broad applications in cell surface protein studies, including the discovery of diagnostic marker proteins and potential therapeutic targets.

2. Experimental procedures

2.1. Reagents and materials

Alexa Fluor™ 568 conjugated streptavidin, EZ-Link™ Sulfo-NHS-SS-Biotin, RPMI 1640 medium, goat serum, and neutravidin agarose resin were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Streptavidin-HRP and HER2/ErbB2 (29D8) antibody were from cell-signaling technology. Antibodies of goat anti-rabbit IgG H&L (HRP), rabbit anti-mouse IgG H&L (HRP), and GAPDH were purchased from Abcam. Bovine serum, protease inhibitor cocktail, trifluoroacetic acid (TFA), dithiothreitol (DTT), iodoacetamide (IAA), sodium chloride (NaCl), ammonium bicarbonate, sodium phosphate dibasic, Triton X-100, Formic acid (FA), and trypsin (bovine, TPCK-treated) were obtained from Sigma (St. Louis, MO, USA). Acetonitrile (ACN, HPLC grade) and methanol (HPLC grade) were from Merck (Darmstadt, Germany). Durashell C18 (L) 5 μm beads were purchased from Agela technologies. 1.9 μm C18-AQ beads were purchased from Dr. Maisch-GmbH (Ammerbuch, Germany). Tween-20, tris, and glycine were purchased from BBI life sciences. C8 membrane was purchased from 3 M Empore™ (St. Paul, MN). Other chemicals and reagents were either of analytical grade or of a better grade. Pure water used in all experiments was purified with a Milli-Q system (Millipore, Milford, MA). The centrifugal filter unit (Amicon® Ultra-0.5 ml) was purchased from Sartorius.

2.2. Cell culture and cell surface biotinylation

Cells were grown in RPMI1640 medium supplemented with 10% fetal bovine serum, 1% penicillin, and streptomycin in a humidified atmosphere of 5% CO_2 in air at 37 °C. Cells of about 80% confluency were subjected to media removal and two washes with ice-cold phosphate-buffered saline (PBS, 0.01 M phosphate, 0.15 M sodium chloride, pH 7.4) followed by a 30-min incubation with EZ-Link Sulfo-NHS-SS-Biotin (0.25 mg/ml in PBS) at 4 °C (Fig. 1). Cells were washed twice with ice-cold tris-buffered saline (TBS, 20 mM tris, 150 mM sodium chloride, pH 7.4) and the residual biotin reagent was blocked with 100 mM glycine in PBS. Next, the cells were harvested by scraping and were pelleted by centrifugation at 500 *g* for 3 min. Finally, the cell pellet was washed twice with TBS and stored at 80 °C.

2.3. Immunofluorescence analysis

After the blocking procedure, HeLa cells were fixed with 4% formaldehyde in PBS at room temperature for 15 min. Then the cells were washed three times with PBS followed by the permeabilization with 0.2% Triton X-100 in PBS for 5 min at room temperature. After three

more washes with PBS, the cells were blocked with 10% goat serum in PBS for two hours, and then incubated with antibodies of 0.2 μg/ml Alexa Fluor™ 568 conjugated Streptavidin in 10% goat serum overnight at 4 °C under the protection from light, which was need for the all following procedures. Subsequently, after four-time washes with PBST (PBS containing 0.05% Tween-20), the cells were then incubated with 1 μg/ml DAPI (4′,6-diamidino-2-phenylindole) at room temperature for one hour. After three washes with PBST, cells were analyzed using the ANDOR™ living cell laser scanning confocal microscope (Revolution WD).

2.4. Cell surface protein isolation

The isolation of cell surface proteins was performed as described by the manufacturer of a Pierce cell surface protein isolation kit with some modifications [9,19]. Cells (15×10^6) were re-suspended in strong RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.2% SDS (*v/w*), 0.5% sodium deoxycholate, 1% Triton X-100, pH 8.0) containing 0.2% protease inhibitor cocktail (*v/v*) and lysed by sonication with 5 × 30 s pulse (Bioruptor® plus sonication device). Subsequently, the resultant cell lysate was centrifuged at 16, 000 ×*g* for 15 min at 4 °C and the protein concentration of the clarified supernatant was determined by BCA assay. Finally, the total cellular proteins were frozen at −80 °C and used for the subsequent affinity purification.

2.5. Western blotting analysis

30 μg of cell lysate was re-dissolved in 1 × non reducing SDS sample buffer before SDS-PAGE, and then the proteins on the gel were transferred onto a PVDF membrane (0.45 μm, Millipore). Subsequently, the membrane was blocked with a blocking buffer of 5% skimmed milk in TBST (0.1% Tween-20, 150 mM NaCl, 20 mM Tris-HCl, pH 7.4) for 2 h at room temperature. Then, the membrane was incubated with HRP-labeled streptavidin diluted 1:4000 in blocking buffer for 1 h at room temperature. After three washes with TBST, HRP substrate (Western Bright, Advansta) was added, and the bands were detected by BioImaging systems (Fusion FX5-XT).

2.6. Affinity purification of biotinylated proteins

For the affinity purification of biotinylated proteins, the classical affinity purification procedure was performed as described by the manufacturer of a Pierce cell surface protein isolation kit with some modifications [9,19]. The details are shown in the Supporting Information. This classical protocol, the elution buffers of which are often containing high concentrations of SDS (2% SDS) is called “SDS, DTT” in the following description. The new optimized isolation protocol was performed as described below. Briefly, 40 μl NeutrAvidin agarose resin was washed three times with PBS and incubated with the cell lysate (1 mg ~ 3×10^6 cells) at room temperature for 1–3 h with end-over-end mixing. After centrifugation at 500 ×*g* for 1 min, the flow-through was discarded, and the beads were carefully washed six times with washing buffer PBS. As for the analysis of 4×10^5, 2×10^5, 10^5 HeLa cells, to reduce the loss of agarose beads during the washing procedure (2.5 μl, 5 μl, 10 μl NeutrAvidin agarose slurry, respectively), we performed the washing procedure in a Gilson 200-μl pipet tip (Fig. S1), as described below. A sieve plate with 1 mm diameter was packed into the Gilson 200-μl pipet tip, and then washed with ACN and PBS, respectively. Next, after the incubation of the sample with NeutrAvidin agarose resin at room temperature for 1–3 h in a 1.5 ml tube with end-over-end mixing, the supernatant was removed and 100 μl of the washing buffer was added. Then the beads were packed into the tip followed by washing with 200 μl PBS six times with a 1-ml syringe as the pressure device (Fig. 5a) [20]. Finally, the beads were transferred to a 2 ml tube and the washing buffer was discarded. Then after the washing procedure, the resultant beads were re-suspended in 100 μl of the elution buffer (PBS, 6 M urea, 0.2% SDS (*v/w*)) containing 50–100 mM DTT and incubated at 65 °C for 30 min to cleave the disulfide bridge in the labeling reagents [21]. The elution procedure was repeated once for complete elution. Besides DTT, the elution buffer is containing the high concentration of urea and this new protocol is called “Urea, DTT” in the following description. In addition, 0.2% SDS in the elution buffer can also be completely removed, which has little influence on the cell surface protein identification (Fig. S2).

Then the eluted proteins were further prepared for LC-MS analysis via FASP (filter-aided sample preparation) protocol using the centrifugal filter unit [22]. Briefly, the collected cell surface fraction was centrifuged at 14000 *g* at 20 °C for 15 min. The flow-through was discarded, 100 μl of 8 M urea in 0.1 M ammonium bicarbonate (NH_4HCO_3, ABC) (UA) was added to the filter unit and centrifuged again. After incubation in the dark with 100 μl of 50 mM IAA dissolved in UA for 40 min at room temperature, the filter was washed once with 100 μl of UA followed by two washes with 0.02 M ABC in water. The proteins were digested with trypsin at an enzyme to protein ratio of 1:25 in 80 μl 0.02 M ABC at 37 °C overnight. The released peptides were collected by centrifugation at 14000 *g* for 10 min followed by washing with 100 μl of 0.02 M ABC once. Finally, the digest was acidified to 3% FA and then was lyophilized for RPLC-MS/MS analysis.

2.7. Hp-RP StageTip fractionation

The high-pH reverse phase (Hp-RP) combined with stop-and-go extraction tip (StageTip) technique was performed as previously reported with some modifications [23]. Briefly, C8 Empore disk was used as a frit inserting into Gilson 200-μl pipet tip. Durashell C18 (L) beads (2.5 mg, 5 μm) were suspended in 100 mM NH_4HCO_3 (pH 10) in 50% ACN and then packed into a Gilson 200-μl pipet tip with a pressure device (Fig. 5a). After sufficient washing and conditioning, cell surface digest was reconstituted in the loading buffer (200 mM NH_4HCO_3, pH 10) and then bound to the StageTip. Next, the cell surface digest was eluted from the tip with increasing concentration of ACN. Detailed procedures of the StageTip fractionation is described in the Supporting Information.

2.8. Nano-RPLC–ESI-MS/MS analysis

The nano-LC-MS/MS experiments were performed using a Dionex UltiMate 3000 RSLCnano system with a Q-Exactive mass spectrometer, controlled by Xcalibur software v2.1.0 (Thermo Fisher Scientific, Waltham, MA, USA). About 2 μg of the lyophilized peptides per sample were re-suspended in 0.1% FA/H_2O solution and automatically loaded onto a 3 cm C18 trap column (200 m i.d) at a flow rate of 5 μl /min. For the capillary analytical column (150 m i.d), one end of the fused-silica capillary was first manually pulled to a fine point as a spray tip and then packed in-house with C18 AQ particles (1.9 μm) to 10 cm. For the RPLC separation, 0.1% FA in water and 0.1% FA in 80% acetonitrile were used as the mobile phase A and B, respectively, and the flow rate was adjusted to ~ 600 nL/min. The gradient of the mobile phase was as follows: for the cell surface digest, from 9% to 35% B in 90 min, up to 45% B in 13 min, up to 90% B in 2 min, where it was maintained for 10 min, up to 4% B in 2 min, where it maintained for 13 min; for the fractionated peptides, from 9% to 45% B in 67 min, up to 90% B in 1 min, where it was maintained for 10 min, up to 4% B in 2 min, where it maintained for 10 min.

The obtained peptides were analyzed by nano-RPLC-electrospray ionization tandem mass spectrometry (RPLC-ESI-MS/MS) in data-dependent MS/MS acquisition mode. The full mass scan acquired in the Orbitrap mass analyzer was from *m/z* 350 to 1750 with a resolution of 70,000 (m/z 200). The 15 most intense parent ions with charge states ≥2 from the full scan were fragmented by higher energy collisional dissociation (HCD). The MS/MS scans were also acquired by the Orbitrap with a 17,500 resolution (m/z 200), and the AGC target was

set to 1×10^5. A spray voltage of 2.6 kV was applied between the spray tip and MS interface. The temperature of the ion transfer capillary was set as 275 °C and the normalized collisional energy for HCD was set 27%. Dynamic exclusion was set as 20 s, and the maximum allowed ion accumulation time was 20 ms for MS scans and 60 ms for MS/MS.

2.9. Data searching and biotin modification identification

All nano-RPLC-ESI-MS/MS raw files were searched against a non-redundant UniProt human database (containing 20,168 sequences, and downloaded from the website of www.uniprot.org) by mascot 2.5 (Matrix Science Inc) implemented on the Proteome Discoverer (Thermo Scientific, v2.1.1.21). Decoy database was generated by Mascot. The parameters were set as follows: trypsin enzyme, maximum 2 missed cleavages, precursor-ion mass tolerance was set to 10 ppm; fragment-ion mass tolerance was set to 0.05 Da, carbamidomethyl (C) as static modification and several dynamic modifications were oxidation (M), acetyl (N term), thioacylation (K and protein N-term) and carbamidomethylthio-propanoylation (K and protein N-term) [24]. The obtained biotinylated sites were further filtered by Best Site Probabilities of ptmRS ≥0.95. The false discovery rate was controlled < 1% in every search result. The other settings were the same as the conventional search. All data were combined from two biological replicates, each with one technical MS run.

2.10. Bioinformatic analysis

Protein cellular localization and statistical enrichment test were analyzed by PANTHER software (http://pantherdb.org/). Proteins, which were unable to be mapped by PANTHER software, were then analyzed by UniProt database (http://www.uniprot.org) for protein cellular localization test. The TMHMM v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and Phobius (http://phobius.sbc.su.se/) were used to predict proteins with the transmembrane domains or a signal peptide. The grand average of hydropathicity (Gravy) values of identified peptides were calculated by the ProtParam program (http://web.expasy.org/protparam/). The exponentially modified protein abundance index (emPAI) values of identified proteins were analyzed by mascot 2.5 (Matrix Science Inc) implemented in the Proteome Discoverer (Thermo Scientific, v2.1.1.21). Label-free quantification (LFQ) analysis was performed using MaxQuant software.

3. Results and discussion

A variety of biotinylation reagents have been developed and applied to the studies of cell surface proteins [25]. Due to its good water solubility and membrane impermeability, the biotinylation reagent of Sulfo-NHS-SS-biotin with a disulfide bridge is very popular for the labeling of cell surface proteins. In this study, we used this reagent and developed efficient protocols for the analysis of cell surface proteome. The workflow is given in Fig. 1. Live cells were incubated with the Sulfo-NHS-SS-biotin and thus proteins on the cell surface were labeled via the reaction of the primary amines with the NHS esters in the reagents. We checked the surface labeling specificity using immunofluorescence assay and found that the biotinylation was only observed on the cell surface (Fig. S1a). This indicated the high specific labeling of cell surface proteins, which also explains the good selectivity of the surfaceome analysis with the reagent. The cells were lysed and the proteins were extracted for affinity purification using avidin beads. Then the captured proteins were eluted from the beads and digested by trypsin for the following LC-MS analysis. Due to the presence of disulfide bridge in this reagent, it can be cleaved in the reducing environment. Thus the biotinylated proteins can be eluted by using reducing reagents like DTT. We also compared the endogenous biotinylation with the labeled biotinylation using western blotting and LC-MS/MS analysis. Negative controls, in which the cells were treated with the same volume of PBS buffer without Sulfo-NHS-SS-Biotin, were prepared along with the experimental groups during all the experimental procedures. The results showed that the endogenous biotinylation was very low and numbers of the identified proteins from the background signal were much smaller compared with these from the positive experiments, which confirmed that the lists of identified proteins were specifically enriched by the affinity purification (Fig. S1b, Table S1, S2).

This workflow is quite straight forward. However, the performance of this method varies a lot depends on the detailed procedures. Weekes et al. compared the techniques to purify plasma membrane proteins. In their work, biotinylation method with the reagent of Sulfo-NHS-SS-biotin gave a modest enrichment and identified 293–434 total proteins from 12×10^7 cells, of which, 78–115 (27–31%) were annotated as plasma membrane proteins [19]. Bennett et al. compared three methods of Sulfo-NHS-SS-biotinylation, aminooxy-biotinylation, and surface coating with silica beads to isolate plasma membrane proteins. Not surprisingly, Sulfo-NHS-SS-biotinylation outperformed the other two methods and yielded the highest absolute number of plasma membrane proteins. In total, 1306 proteins were identified, of which, 650 (49%) were annotated as plasma membrane proteins from 3×10^7 cells [8]. In this study, we optimized the classical workflow of biotinylation method to improve the number of identified cell surface proteins from the reduced amount of starting cells. In addition to the reducing reagent to cleave the disulfide bonds, other components in the elution buffer, especially the detergents, are also critical to its performance. SDS, especially at high concentrations, is highly effective in the solubilization of membrane proteins [26] and is often added in the elution buffer for the highly hydrophobic cell surface protein analysis [16]. However, SDS must be removed before the protein digestion and LC-MS/MS. Otherwise, the residual SDS will result in the inhibition of trypsinization and the contamination of LC columns, leading to poor identification performance. In the early days, the elution buffer with SDS was often used to elute the cell surface proteins from the avidin beads followed by gel electrophoresis [16]. The SDS was then removed after electrophoresis, so it has no interference on in-gel digestion. However, this method is time-consuming [27] and has poor sensitivity to identify integral membrane proteins, especially integral membrane proteins with multiple transmembrane segments [28]. More recently, the development of FASP has greatly facilitated the removal of detergents and FASP combined with the strong detergent of SDS has been extensively applied to the digest of membrane proteins [22,28]. However, high concentrations of SDS in protein elution buffer normally need long washing time for completely removing, which in turn increase the protein loss.

In this study, we first evaluated the performance of cell surface biotinylation method using the classical protocol with the elution buffer containing high SDS fraction. We identified a total of 400 proteins using elution buffer containing 2% SDS and 50 mM DDT. Then the proteins were analyzed by PANTHER software and UniProt database. In many cell surface protein identification studies [8,9,18,19,29], proteins with Gene Ontology cellular component (GO.CC) annotations of “plasma membrane”, “cell surface”, or “extracellular” (including the terms of “extracellular matrix”, “extracellular region”, “extracellular space”, “extracellular vesicle”, and “extracellular exosome”) were termed as cell surface proteins [9,18,29] or plasma membrane proteins [8,19] to evaluate the performance of cell surface protein capture approaches. This is because these represent surface-exposed proteins were targeted by the extraction methods [8]. These proteins were termed as cell surface-associated proteins in this study. Among the 400 proteins identified, 328 (82%) proteins were annotated as cell surface-associated proteins (Fig. 2a). The specificity is excellent but the identification number is small, which may result from the residual SDS in the eluted sample. Therefore, we took a long time to remove SDS by repeatedly washing the eluted proteins with 8 M urea buffer on the filter using FASP, which resulted in the identification of 2138 proteins, among

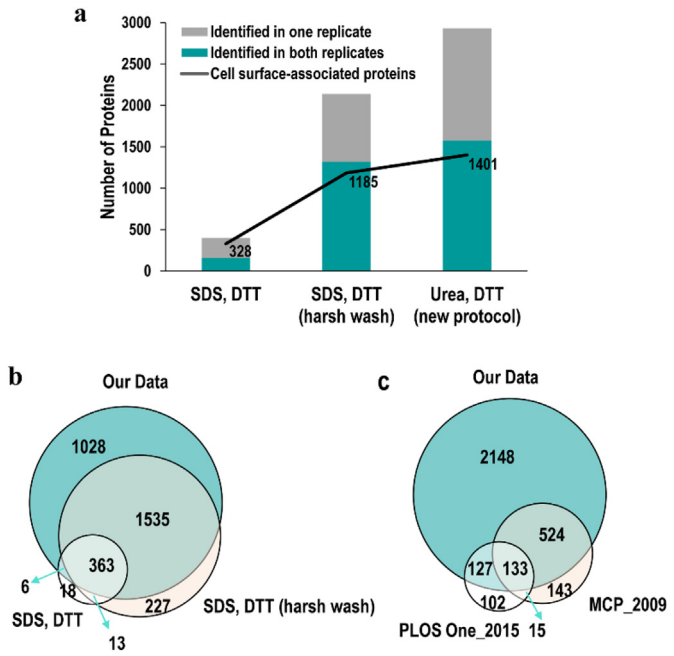


Fig. 2. The performance of cell surface-associated protein identification for the new protocol. (a) The numbers of cell surface-associated protein identification by different protocols (combining data from two biological replicates, each with one technical MS run. The "Urea, DTT" label refers to the new protocol). (b) Proteins identified by the new protocol can cover about 88.8% of the proteins identified by the classical workflow with harsh washing procedure. (c) Our data can cover > 75% of the two published HeLa cell surface proteome data [10,32].

which, 1185 (55.4%) proteins were annotated as cell surface-associated proteins (Fig. 2a). The numbers of total proteins and cell surface-associated proteins increased 5.3 and 3.6 folds, respectively. And this number of total proteins was used as the final identification result of the classical protocol in the following sections for comparison. Clearly, this harsh washing step in FASP significantly improved the cell surface protein identification. However, the harsh washing procedure is tedious, which typically takes 3 to 5 h, and may also result in more protein loss (Fig. 3a).

Strong chaotropic reagents such as urea, are often used in MS-proteomics studies due to their compatibility with proteases and easy removal. However, it has long been recognized that the urea solution can cause the carbamylation of proteins/peptides, which is accelerated at elevated temperature and prolonged incubation time [30]. Thus, at the beginning of our experiment, we firstly evaluated the extent of urea-induced carbamylation under our sample preparation condition. Carbamylation (N-terminal, K; +43 Da) was additionally set as dynamic modification during database searching. As seen in the Table S3, though the proportions of carbamylated peptides under our sample preparation conditions were a bit higher than these from whole cell lysates, all of the proportions of carbamylated peptides were small (< 1.7%). Therefore, we used the elution buffer with high concentrations of urea (6 M urea) and low concentrations of SDS (0.2% SDS), which, if necessary, can also be completely omitted (Fig. S2), to release the proteins from the avidin beads [21]. As a result, more cell surface proteins were identified with good selectivity, sensitivity, and reproducibility. A total of 2932 proteins were identified and 1401 (47.8%) proteins were annotated as cell surface-associated proteins (Fig. 2a, Table S4). Additionally, 88.8% (1898) of the proteins identified by the classic protocol with harsh washing procedure were also identified by the new protocol (Fig. 2b), indicating that the urea containing elution buffer can effectively extract the cell surface fraction. Then, to further compare the enrichment of cell surface proteins with both protocols, emPAI values of proteins identified with both protocols were analyzed. The emPAI is roughly proportional to protein abundance and can be applied to quantitative proteomics analysis [31]. As seen in Fig. 3a, for most of the identified proteins, the new protocol had larger emPAI values and thus less protein loss. Compared the results with two published HeLa cell surface proteome data (Fig. 2c), our coverage, in term of identified cell surface-associated proteins, increased about 3.7 and 1.7 folds, respectively [10,32]. In addition, the majority of their identifications were also covered by our data. As seen in Fig. 2c, our data can cover about 69.0% (260) of the HeLa cell surface proteins identified in the database of recently assembles "Cell Surface Protein Atlas" by the CSC technology [10] and about 80.6% (657) of cell surface proteins identified by combining the methods of lectin affinity chromatography and glycocapture approaches [32]. We also compared the performance of protein identification between this method with the global proteomics approach where total cell lysate was analyzed. It can be seen from Table S5 and Fig. 3b, the emPAI values, sequence coverages and the spectra counts for the cell surface-associated proteins have been greatly improved in the new protocol compared with total cell lysate approach. For example, the spectra count for EGFR, an important receptor protein on the cell surface, increased from 13 to 222, increased by about 17 folds. And its sequence coverage also increased by about 4.8 folds.

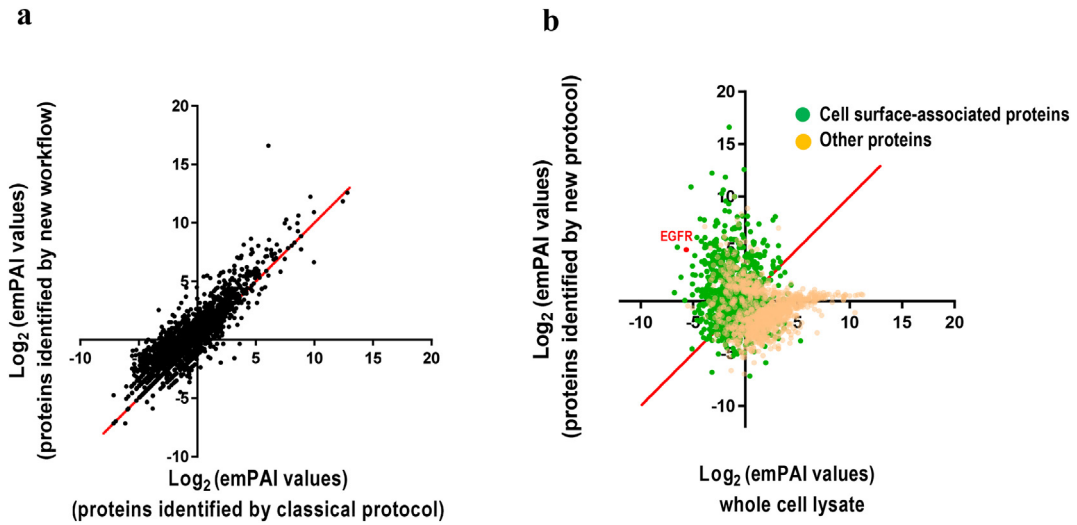
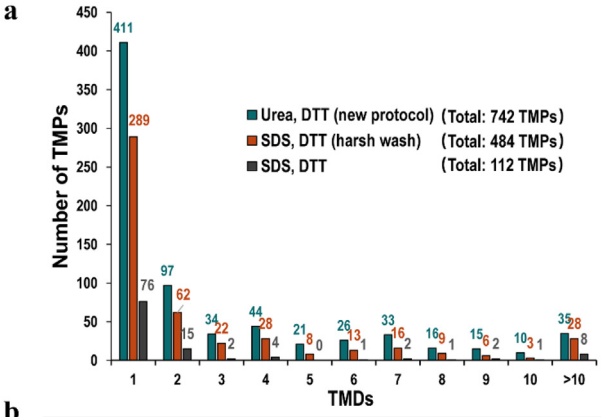


Fig. 3. Comparison of the abundance of the proteins enriched by (a) the new protocol and classical protocol; (b) the new protocol and the total cell lysate. The emPAI values were used to estimate absolute protein abundance.

Clearly, this cell surface proteome labeling approach significantly improved the coverage. Then, to assess the reproducibility of the protocol, LFQ analysis between replicate experiments was performed using MaxQuant software. Two biological, two sample preparations, and two LC-MS run replicates were performed and then LFQ values were plotted against each other replicate using the Perseus software (Fig. S3). It is no surprise that the two LC-MS runs have the highest correlation coefficient ($R^2 > 0.994$). However, the two biological replicates and the sample preparation replicates also showed high reproducibility with a good correlation coefficient ($R^2 > 0.93$). This indicated that this new protocol of cell surface biotinylation method has good reproducibility. Finally, statistical enrichment tests of our data and the whole cell lysate were performed according to GO terms in the domain of GO cellular component [33] (Table S6, S7) and all the genes in the PANTHER database for *Homo sapiens* were set as the reference gene list by default. Significant enrichment for "cell surface" proteins (p-value < .0000000005) was observed and 208 "cell surface" proteins from HeLa cell line were identified in our data (total 909 "cell surface" proteins from all the genes in the PANTHER database for *Homo sapiens* and 99 "cell surface" proteins from the result of total HeLa lysate). Besides, compared with the results of whole cell lysate, "GPI-anchor transamidase complex" proteins, "receptor complex" proteins and extracellular proteins, including "extracellular matrix", "laminin complex", "protein complex involved in cell adhesion", "basement membrane" et al., also showed significant enrichment (p-value < .05), demonstrating that the labeling reagents were targeted on all the cell surface-exposed proteins.

The proteins labeled with sulfo-NHS-SS-biotin probe carry a carbamidomethylthio (CAM)-propanoyl or thioacyl modification which can be identified and then help us confirm the non-cell-membrane-permeability of the sulfo-NHS-SS-biotin reagent by the localization of labeled proteins identified [8]. In this study, as seen in Table S8, we identified 544 labeled sites in 222 proteins from HeLa cells (Table S9), which was better than the number (245 labeled sites in 108 proteins) identified by the classic protocol with harsh washing procedure (Table S10). It was found that about 90% of biotinylated proteins were annotated as cell surface-associated proteins and 10 of these another 23 were proteins annotated by UniProtKB/Swiss-Prot as "integral component of membrane" or "membrane". These sites information could be helpful to find genetically- or drug-induced changes, or to analyze cell surface protein topologies.

CD (clusters of differentiation) antigens are cell surface molecules providing targets for immune-phenotyping of cells. They are commonly acting as receptors or ligands and used as cell markers. More recently, they have been recognized as invaluable tools for the treatment of several malignancies and autoimmune diseases [6]. Up to now, CD for humans is numbered up to 408 (as of 22 January 2016) [34]. In this work, a total of 91 CD antigens (corresponding to 93 cell surface proteins) from HeLa cells were identified by the new protocol (Table S5, S11), indicating the high efficiency of our new protocol for cell surface protein enrichment. To further evaluate the performance of our enrichment approach, we predicted the numbers of the identified proteins with the transmembrane domains (TMDs) and a signal peptide (SP) using software of TMHMM and Phobius. As seen in Fig. 4b, the proportions of proteins containing TMDs or SP from our new protocol were much higher than that from the whole cell lysate, which further confirmed the selectivity of our new protocol. Meanwhile, we also compared the identified proteins with the predicted TMDs from different protocols of cell surface biotinylation. As seen in Fig. 4a, 742 out of 2932 (25.3%) by the new protocol, 484 out of 2138 (22.6%) by the classic protocol with harsh washing procedure, 423 out of 2884 (14.7%) for whole HeLa cell lysate were predicted to be transmembrane proteins (TMPs). Besides, Gravy values, molecular weights (MWs) of the identified proteins were also analyzed. The results showed that among the 2932 proteins identified, 534 (18.2%) had MW > 100 kDa and 330 (11.3%) had Gravy > 0, and these numbers for the whole cell lysate were 416 (14.4%) and 232 (8.04%), respectively (Fig. S4). The results



b

Methods (HeLa cell line)	Urea, DTT (new protocol)					Total cell lysate
Number of starting cells ($\times 10^5$)	1	2	4	10	40	
Total proteins IDs	731	1211	1555	2932	4511	2883
TMD alone (%)	240 (32.8)	396 (32.7)	481 (30.9)	742 (25.3)	1065 (23.6)	423 (14.7)
SP alone (%)	224 (30.6)	334 (27.6)	380 (24.4)	578 (19.7)	768 (17.0)	334 (11.6)
TMD and SP (%)	309 (42.3)	508 (42.0)	615 (39.5)	991 (33.8)	1389 (30.8)	618 (21.4)
Neither TMD and SP (%)	422 (57.7)	703 (58.0)	940 (60.5)	1941 (66.2)	3122 (69.2)	2265 (78.6)

Fig. 4. Comparison of the numbers of the identified proteins with the transmembrane domains (TMDs) and a signal peptide (SP) from HeLa cells using the software of TMHMM and Phobius. (a) The numbers of the identified proteins with different TMDs. (b) The numbers of the identified proteins by the new protocol with TMDs and/or SP were presented. The surface proteomes from 4×10^6 HeLa cells were identified by combining with the rapid Hp-RP StageTip fractionation scheme. All data were combined from two biological replicates, and each with one technical MS run.

showed that the urea containing elution buffer can effectively extract the hydrophobic cell surface proteins and the new protocol tended to capture cell surface proteins with more hydrophobic transmembrane domains.

At present, almost all methods for the enrichment of cell surface proteins require large amounts of starting material (10^7 to 10^8 cells per experiment), which may not always be available, such as endogenous stem/progenitor cells. To enable this new protocol to analyze a small number of cells, further optimization was performed. As shown in Fig. 5a, to reduce the loss of avidin agarose during the washing procedure, we performed the washing procedure in a Gilson 200-μl pipet tip with a 1-ml syringe as pressuring device (Fig. 5a). For 4×10^5, 2×10^5, 10^5 HeLa cells, we identified 916, 791, 547 cell surface-associated proteins with high selectivity, respectively (Fig. 4b and 5b). Not surprisingly, the number of identified cell surface-associated proteins decreased with the reducing of cell number but the specificity of the new protocol increased. To analyze cell number lower than 10^5, the amount of agarose may need further decreasing, which is not easy to perform. Thus our current protocol may be not applicable to the cell number lower than 10^5. Herein, this new workflow for the cell surface biotinylation method is applicable to a very small number of cells (10^5 to 10^6 cells per experiment), such as stem/progenitor cells.

Fractionation of the peptides is an effective approach to reduce sample complexity and improve the analysis coverage [35]. Nurhan Özlü et al. performed the IEF (Isoelectric focusing)-based fractionation for the cell surface digest obtained by the biotinylation method with the reagent of sulfo-NHS-SS-biotin, which was then analyzed by LC-MS. The experiment was repeated five times. As a result, 4278 proteins, including 628 GO-annotated cell surface protein groups, were identified in total and average 23.7% of the identified proteins for each

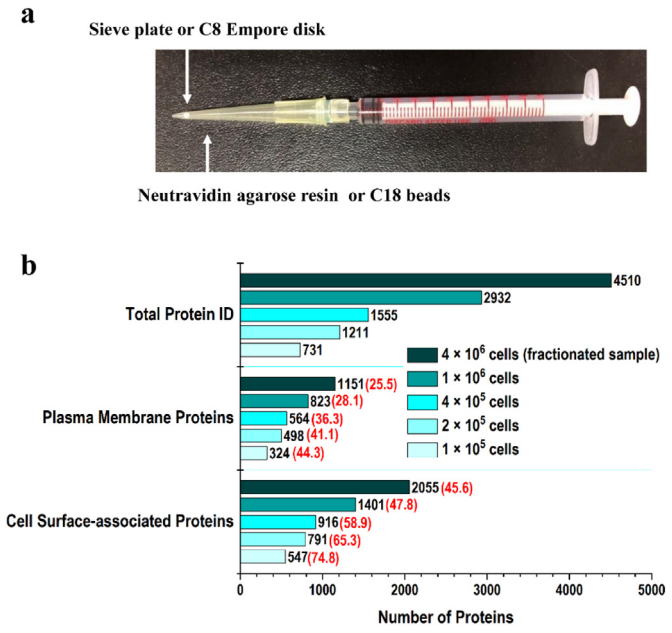


Fig. 5. Sensitive and comprehensive analysis of the cell surface proteins by using a pipet tip. (a) A Gilson 200-μl pipet tip with a 1-ml syringe as the pressure device was made to reduce the loss of agarose rein for the analysis of a small number of cells and rapid Hp-RP StageTip fractionation for in-depth cell surface proteome analysis. (b) Comparison of the proteins identified with different amounts of starting cells. The Hp-RP StageTip fractionation was only applied to analyze ~ 4×10^6 cells.

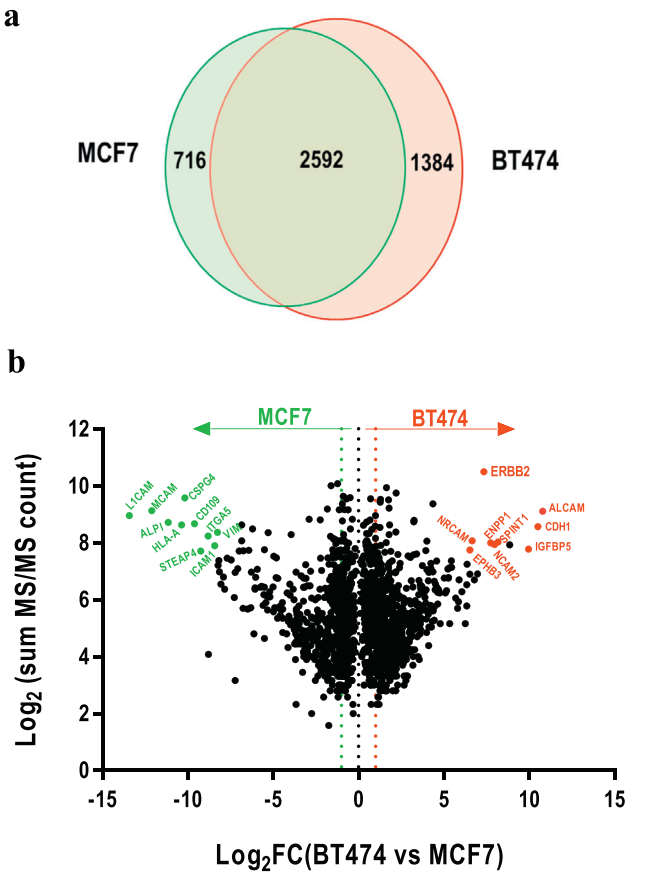


Fig. 6. The proteins identified from BT474 and MCF7 cell lines were quantified based on the label-free quantitative MS using Perseus software. (a) Venn diagram of total proteins identified from each cell line. (b) The volcano plot of the differential proteome analysis between BT474 and MCF7 cell lines (p-value < .01). Y-axis is spectra count reflecting the protein abundance.

experiment was annotated as plasma membrane proteins by GO annotation in HeLa cells [29]. In this paper, to enable fractionation of minute amount of sample, C18 particles were placed in 200-μl pipet tip and a 1-ml syringe with pistons was used for washing and elution (Fig. 5a). By using this device, the fractionation of peptides by using high pH buffers could be completed in only 15 min. The enriched HeLa cell surface digest (20 μg, from ~ 4×10^6 cells) was fractionated into six fractions. And then each fraction was analyzed by LC-MS/MS. It can be seen in Fig. 5b, 4510 proteins were identified, of which, 1151 (25.5%) and 2055 (45.6%) proteins were annotated as plasma membrane and cell surface-associated proteins, respectively. Much higher specificity and much more cell surface proteins were obtained compared with the Nurhan Özlü's study. We compared the identifications between the adjacent fractions and found only about 6% peptides common identified. This indicated the high resolution of the fractionation. The deep coverage of this analysis can also be evidenced with the facts of 1065 transmembrane proteins and 103 CD antigens (corresponding to 107 cell surface proteins) were identified (Fig. 4b, Table S12). To our knowledge, this is the most comprehensive proteomic analysis of cell surface proteins in HeLa cells to date [31]. Bring together, the rapid Hp-RP StageTip fractionation approach presented here can be used for in-depth cell surface proteomic profiling.

Most signaling pathways are initiated by the engagement of cell surface receptors. A better understanding of the cell surface proteome in cancer cell lines can help us discover the disease-specific biomarkers and potential therapeutic targets. In this study, we analyzed the cell surface proteins of two well-characterized breast cancer cell lines, BT474 and MCF7 [36,37], by the new protocol of biotinylation method. Totally 3975 and 3308 proteins were identified from BT474 and MCF7, respectively (Fig. 6a). Among them, 1565 and 1478 were annotated as cell surface-associated proteins. The surface proteomes identified in each of the 2 biological replicates were quantified by the label-free quantification approach using the Perseus software. It was found that all replicates have a good correlation coefficient (R^2 > 0.93, Fig. S6), indicating good reproducibility. Between these two cell lines, 1482 proteins, including 774 cell surface-associated proteins, were found to be significantly different (p-value < .01) after applying a Benjamini-Hochberg method (Table S13). Many proteins described in the literature as putative cancer biomarkers proteins, such as proliferating cell nuclear antigen (PCNA), S100 proteins (S100A4, S100A14, S100A16, S100A10), E-cadherin, 14-3-3-sigma, antigen Ki-67, calreticulin, cathepsin B [38–40], were also identified in our study and were found to be differentially expressed. The high abundant specifically expressed proteins could be used to classify different cells. The volcano plot shows that many high abundance proteins (with high spectra counts) were differentially expressed in BT474 and MCF7 (Fig. 6b). Among them, Her2 (ERBB2) was found to be almost exclusively expressed in the BT474 cell line with the fold of 162. This is consistent with the characteristics of these two cell lines [41] and was validated by western blot (Fig. S5). Above analysis revealed the high heterogeneity of breast cancers. The label-free quantitative surfaceome analysis would provide a unique molecular fingerprint to classify cells and provide important information to guide immunotherapy.

Our method is extremely sensitive as evidenced by the fact that 4510 proteins including 2205 annotated as cell surface-associated proteins were identified from only 20 microgram protein digest. With so many proteins identified, one may question how many proteins really present on the cell surface. Immunofluorescence assay indicated that the biotinylation was only observed on the cell surface (Fig. S1a) and so the labeling was highly specific to cell surface proteins. However, the identification of non-cell surface proteins is possible and it could be due to other reasons. Firstly, non-cell surface proteins were co-purified through strong noncovalent interactions with hydrophobic

cell surface proteins. Secondly, a small part of the cells may be disrupted during the labeling procedure. Indeed, some intracellular proteins, such as transcription intermediary factor 1-beta, transmembrane protein 41B, LIM, and SH3 domain protein 1, were also present in our results. Besides, some non-cell surface annotated proteins may be located on the cell surface, but not yet annotated as such. Theoretically, cell surface proteins will be enriched but non-cell surface proteins will be reduced or completely removed by cell surface biotinylation method. Thus the cell surface proteins should be presented as high abundance one. The numbers of peptide-spectrum matches (PSMs) roughly reflect the abundance of proteins. As shown in Fig. S7, the specificity, i.e. the percentage of annotated cell surface-associated proteins, increased with the increasing of PSMs numbers. Clearly, the proteins identified with high numbers of PSMs are more likely to be bona fide cell surface proteins. Nevertheless, for a specific protein, further validation experiment is required.

4. Conclusion

In summary, we optimized the cell surface protein isolation protocol with improved cell surface protein coverage. The new protocol is easier to perform and time-saving. It has high selectivity, sensitivity, and reproducibility. With a special pipet tip, the new workflow is applicable to a very small number of cells and about 600 cell surface-associated proteins were identified from only 1×10^5 cells. Besides, the rapid Hp-RP StageTip fractionation scheme enabled the identification of 2055 cell surface-associated proteins from 4×10^6 cells. To our knowledge, this is the largest dataset obtained for cell surface proteins of HeLa cells. The new protocol was applied to the differential analysis of cell surface proteomes of two well-characterized breast cancer cell lines, BT474 and MCF7. Many cell surface-associated proteins were found to be differentially expressed between these two breast cell lines. This information could be valuable to reveal the heterogeneity of breast cancers.

Acknowledgments

This work was supported, in part, by funds from the National Key R &D Program of China (2017YFA0505003, 2016YFA0501402), the National Natural Science Foundation of China (21605140, 21535008, 21405516, 21775146, 81600046, 81430072), CAS-Weigao Research & Development Program ([2017]-009), the innovation program (DICP TMSR201601)of science and research from the DICP, CAS. M.Y. is a recipient of the National Science Fund of China for Distinguished Young Scholars (21525524).

Conflict of interest

All authors would like to confirm that there is no any conflict of interest with this research and manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2019.01.015.

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