Proteomic Analysis of Cell Surface Proteins with Improved Specificity of Enrichment

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ABSTRACT

Purpose: Enrichment of cell surface proteins is commonly performed by biotinylation using amine-based chemistry; however, this method is often associated with intracellular labeling and difficulty distinguishing the true cell surface proteins from background contaminants. Here, we have developed a robust cell surface protein isolation method using amine-based chemistry with reduced background that is compatible with mass spectrometry.

Methods: A new cell surface labeling protocol (Figure 2) was compared with the Thermo Scientific™ Pierce™ Cell Surface Protein Isolation Kit. In both protocols, mammalian cells were labeled with Sulfo-NHS-SS-Biotin (see Figure 1). Following removal of the label, the cells were washed, harvested, and lysed in detergent. Biotinylated proteins were captured on NeutrAvidin agarose, washed and eluted with reducing agent (DTT). Western blots were performed to identify specific cell surface proteins and intracellular contaminants. Alternatively, captured proteins were eluted with DTT from washed resin, alkylated and then digested with a Trypsin /Lys-C Protease mix. Peptides were cleaned up and then analyzed by LC-MS using label free quantification on a Thermo Scientific™ Q Exactive™ Plus platform.

Results: A revamped method for cell surface protein enrichment with Sulfo-NHS-SS-Biotin was successfully developed using modified reagents and an improved protocol, resulting in better selectivity of cell surface targets.

INTRODUCTION

Cell surface proteins are a rich source of therapeutic targets for disease and play a major role in signal transduction, cell adhesion and ion transport. Cell surface biotinylation is a popular chemical enrichment method for the cell surface proteome. A reactive biotin ester is used to label the extracellular domains of integral plasma membrane proteins, commonly through primary amines. Following cell lysis, the labeled proteins are captured using biotin-binding supports. This is an efficient method for cell surface protein isolation, but there is a tendency for the reactive biotin to cross the cell membrane and label intracellular proteins, thereby contaminating the enriched cell surface targets. In this study we significantly reduced intracellular protein labeling with targeted protocol improvements.

MATERIALS AND METHODS

Sample Preparation (see Figure 2)

HeLa, A549 and HCT-116 cell lines (ATCC) were cultured in Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum in 15cm dishes to 85-95% confluency. One dish was used per sample. Cells were labeled with 10mL of 0.25mg/mL Sulfo-NHS-SS-Biotin in phosphate buffered saline (PBS) for 10min at room temperature (RT). Label was removed and the cells were washed with Tris buffered saline (TBS). Cells were scraped from the plate into TBS and pelleted at 500 x g for 3 minutes. Cell pellets were lysed in 500µL of Lysis Buffer for 30 minutes on ice. Clarified lysate was incubated for 30 minutes with 250µL of NeutrAvidin agarose (50% slurry) at RT to capture biotinylated proteins. The agarose was washed with Wash Buffer and then proteins were eluted with 10mM DTT for 30 minutes at RT (Western blotting) or the resin was washed 3 times with 500µL 0.1M triethylammonium bicarbonate (TEAB) and eluted with 10mM DTT for 45 minutes at RT (MS analysis). Subsequent MS sample prep was performed using the Thermo Scientific™ EasyPep™ Mini MS Sample Prep Kit. Briefly, eluate was alkylated and then digested with a Trypsin /Lys-C Protease mix. Peptides were cleaned up and resuspended in 0.1% formic acid in water.

Test Methods

For Western blot analysis, eluates were mixed with sample buffer, resolved on 4-20% Tris-Glycine gels, and transferred to nitrocellulose using an Invitrogen™ Power Blotter XL System. Blots were blocked using Thermo Scientific™ StartingBlock™ T20 (TBS) Blocking Buffer, incubated with primary antibody (as indicated) against either cell surface proteins or intracellular proteins for 1hr, washed with TBS containing 0.05% Tween-20 (TBST), and then incubated with secondary antibody conjugated to Horseradish peroxidase for 30 minutes. After washing with TBST, bands were detected using Thermo Scientific™ SuperSignal™ West Dura Extended Duration Substrate and an Invitrogen™ iBright™ FL1000 Imaging System.

For MS analysis, peptides were analyzed by LC-MS using label free quantification on a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer.

Data Analysis

Raw data was searched using Thermo Scientific™ Proteome Discoverer™ Software. Plasma membrane proteins were identified using the list of human plasma membrane proteins in the Uniprot Knowledgebase (UniProtKB).

RESULTS

Isolation of cell surface proteins

The Pierce Cell Surface Protein Isolation Kit (no. 89881) was improved to reduce intracellular labeling and background contamination in the enriched fraction. Both the new and old protocols use Sulfo-NHS-SS-Biotin as the labeling reagent (Figure 1). This chemical modifies primary amines (primarily lysine residues) on cell surface proteins. The biotin on the linker allows for capture of the modified proteins onto NeutrAvidin agarose, and the disulfide bond in the spacer arm allows for detachment of the bound proteins using reducing agent (DTT). Figure 2 contains a schematic of the revised protocol. It differs from the original protocol in several ways. The new protocol removes the label instead of quenching it; the latter was found to cause intracellular labeling during the cell harvest. In addition, to improve efficiency, the amount of label per reaction was reduced by 50%, incubation times were all reduced by 20 to 30 minutes, the lysis buffer was updated with a detergent that solubilizes multi-spanning membrane proteins more efficiently, and MS analysis was added to western blotting as the compatible applications. For the former, detergents used in lysis and wash steps were modified and/or removed so as to prevent interference with LC/MS. All of these changes resulted in significantly reduced background contaminants along with similar to higher yield of cell surface proteins with HeLa cells (Figure 3).

Figure 1. Sulfo-NHS-SS-Biotin labeling reagent.

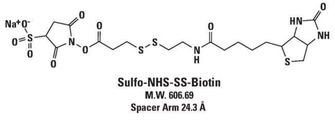


Figure 2. Procedure summary for the Pierce Cell Surface Protein Biotinylation and Isolation Kit (Product No. A44390).

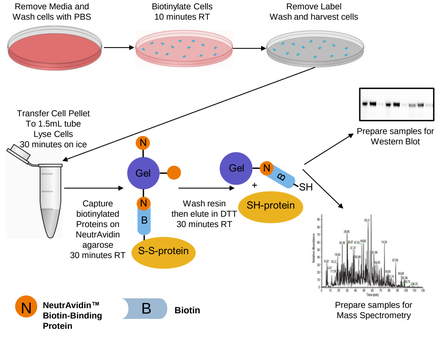
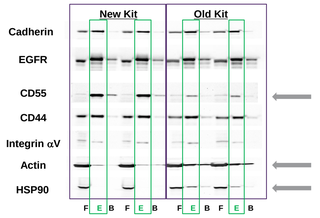


Figure 3. The new method improves yield of cell surface proteins and significantly reduces background compared with the old method. Samples were prepared in duplicate from HeLa cells using the new isolation method described in Methods and the old kit (Thermo Fisher Scientific #89881) and then normalized by volume. Flow-through (F), elution (E) and bead boil (B) fractions were analyzed by Western blot for cell surface proteins (EGFR, CD55 and Cadherin, CD44 and Integrin αV) or intracellular proteins (HSP90 and Actin).



MS analysis of New vs Old cell surface protein isolation methods

Labeled cell surface proteins from A549 cells were isolated according to either the new or old method on NeutrAvidin resin, washed with TEAB, and eluted with 10mM DTT. After alkylation, digestion and clean-up, the samples were normalized by peptide amount and then analyzed by LC-MS. Proteins were identified as plasma membrane (cell surface) using UniProtKB. Samples were prepared in duplicate. The number of proteins identified as cell surface was 27% higher with the Old method vs the New method; however, the background contaminants were 3-fold higher with the Old method (Figure 4). As a result, cell surface proteins were detected in lower abundance with the old method due to the upper limit of sample amount that can be analyzed by MS (1-2ug on a column) (Figure 5). In contrast, selected cytosolic contaminants were found to be 2- to 7-fold higher in abundance with the old method (Figure 6, A and B). Comparison of the top 100 most abundant proteins for both methods identified 5-fold more cell surface proteins and 4-fold fewer intracellular proteins with the new method (Figure 7). The protein distribution for the old method was similar to an A549 cell lysate.

Figure 4. Higher number of cell surface proteins identified with the Old method, but with significantly more contaminants compared with the New method.

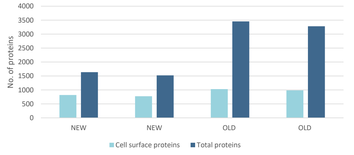


Figure 5. Higher abundance of selected cell surface proteins with New vs Old methods.

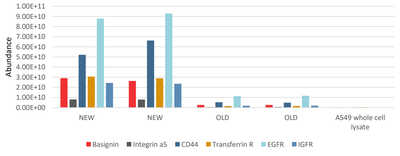


Figure 6. Higher abundance of cytoplasmic tubulins (A) and Actin (B) with Old vs New methods.

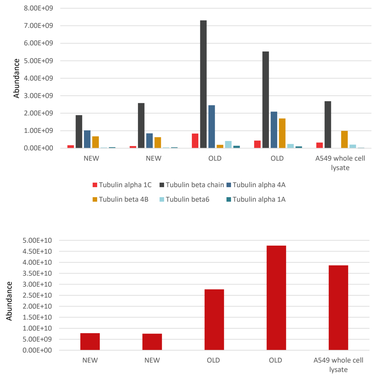
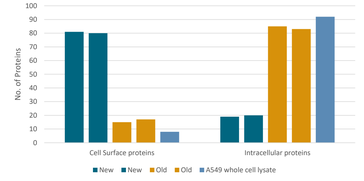


Figure 7. Classification of top 100 most abundant proteins identified by MS using the UniprotKB. Proteins were manually reviewed in the database to confirm categorization.



MS analysis of cell surface enrichment with 3 cell lines

Cell surface proteins were enriched from 3 cell lines, HCT-116, A549 and HeLa, using the Method in Figure 2. Following MS sample preparation, the peptide yield was determined using the Pierce™ Quantitative Colorimetric Peptide Assay Kit and found to be similar across the 3 cell lines (10-14µg peptides) (Table 1). A control sample was prepared using the old method and found to yield significantly more peptides (92µg), the majority being intracellular proteins (Figure 4). Using the new method, replicates of each cell line were prepared using one plate of confluent cells per sample, and then analyzed by MS. Comparing the 3 cell types, approximately 55-60% of the total identified proteins and 86-92% of the top 100 most abundant proteins were identified as cell surface proteins (Figure 8). The top 50 most abundant proteins were manually reviewed in the UniprotKB, and 92-95% were confirmed to be cell surface proteins (Figure 9). Lastly, the types of enriched cell surface proteins were further evaluated using the UniprotKB, classifying them as single- or multi-spanning (6-17 spans) transmembrane (TM) proteins, glycosylphosphatidylinositol (GPI) anchored proteins, and extracellular matrix (ECM) proteins (Figure 10). A similar distribution was seen across all three cell lines.

Table 1. Peptide yield of isolated cell surface proteins processed by MS sample prep

Cell Line	Method	Peptide yield per 15cm dish of 85-95% confluent cells (µg)
HeLa	New	10.8 ± 0.9
HCT-116	New	11.4 ± 2.6
A549	New	13.7 ± 0.3
A549	Old	91.9 ± 12.0

Figure 8. Percentage of cell surface proteins isolated out of total proteins identified

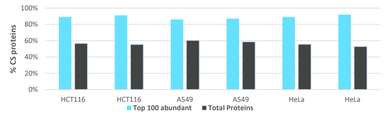


Figure 9. Comparison of the number of cell surface and intracellular proteins in the top 50 most abundant proteins identified by MS analysis

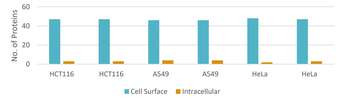


Figure 10. Types of enriched cell surface proteins in the top 50 most abundant proteins identified by MS analysis

CONCLUSIONS

- An improved method for isolation of cell surface proteins using amine biotinylation resulted in 3-fold fewer intracellular contaminants than the original method, allowing for improved selectivity and enrichment of cell surface proteins.
- The method and reagents for cell surface protein isolation were modified to allow compatibility with mass spectrometry.
- Cell surface proteins, including single- and multi-spanning transmembrane proteins and GPI anchored proteins, were enriched from three cell lines.
- Cell surface biotinylation can be used to identify potential cell surface antigens, to monitor changes in cell surface proteins in response to small molecule treatments, or mapping exposed surface domains of membrane proteins.

TRADEMARKS/LICENSING

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PO65558-EN0519S