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An overview on enrichment methods for cell surface proteome profiling

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Cell surface proteins are essential for many important biological processes, including cell–cell interactions, signal transduction, and molecular transportation. With the characteristics of low abundance, high hydrophobicity, and high heterogeneity, it is difficult to get a comprehensive view of cell surface proteome by direct analysis. Thus, it is important to selectively enrich the cell surface proteins before liquid chromatography with mass spectrometry analysis. In recent years, a variety of enrichment methods have been developed. Based on the separation mechanism, these methods could be mainly classified into three types. The first type is based on their difference in the physicochemical property, such as size, density, charge, and hydrophobicity. The second one is based on the bimolecular affinity interaction with lectin or antibody. And the third type is based on the chemical covalent coupling to free side groups of surface-exposed proteins or carbohydrate chains, such as primary amines, carboxyl groups, glycan side chains. In addition, metabolic labeling and enzymatic reaction-based methods have also been employed to selectively isolate cell surface proteins. In this review, we will provide a comprehensive overview of the enrichment methods for cell surface proteome profiling.

KEYWORDS

cell surface proteins, enrichment methods, plasma membrane proteins, proteomics

1 | INTRODUCTION

Cell surface physically defines the boundary between each cell and its environment, and proteins on the cell surface are directly involved in many important cellular events, including cell–cell interaction, signal transduction, cell adhesion, and protein trafficking [1,2]. Cell surfaces are dynamic structures, and many cell surface proteins function as receptors, transporters, carriers, channels, cell-adhesion proteins, and enzymes, in response to ever-changing extracellular environment by triggering downstream signaling events [3–5]. Except their crucial rules in biological functions, cell surface proteins account for nearly two-thirds of the protein-based drug targets and many cell surface proteins serve as markers to help the classification and isolation of specific cell types, enabling the linkage of distinct proteotypes to functional phenotypes [6–8]. In the past decade, many efforts have been focused on the discovery of specific surface markers for embryonic stem cells and crucial antigens, which are involved in host–pathogen interactions, for vaccine development [1,9–18]. More recently, cell surface protein-directed monoclonal antibodies (mAbs) have been found to be greatly valuable for the clinical treatment of cancers, autoimmune diseases, etc. [1,19,20]. Thus, characterizing the cell surface proteins and their dynamic regulation is critically important for the understanding of many cellular processes and diseases.

Article Related Abbreviations: CCS, cationic colloidal silica; CSC, cell surface capturing; FFE, free-flow electrophoresis; HRP, horseradish peroxidase; LFQ, label-free quantification; NHS, *N*-hydroxysuccinimide ester; PM, plasma membrane; PMP, plasma membrane protein; PNGase F, peptide *N*-glycosidase (PNGase) F; PTM, post-translational modification; SILAC, stable isotope labeling by amino acids in cell culture; TMP, transmembrane protein; TMT, tandem mass tag; WGA, wheat germ agglutinin; 2PAP, two-phase affinity partitioning; 2PP, two-phase partition.

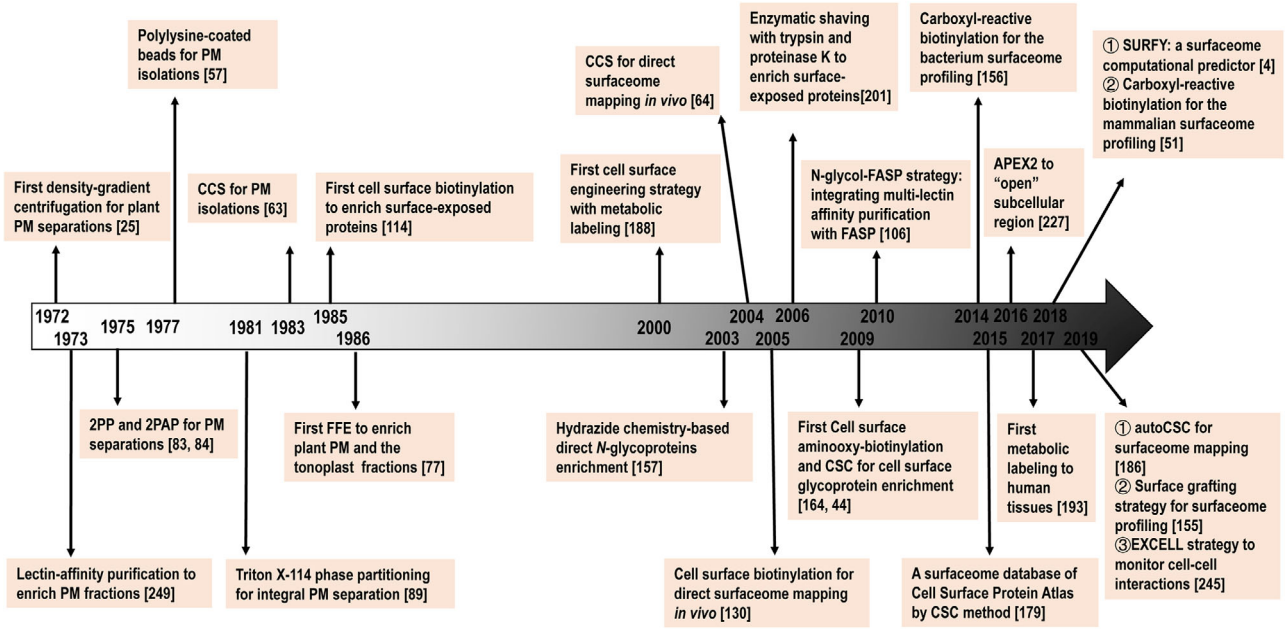

FIGURE 1 A brief timeline of the development history of cell surface protein enrichment methods. In recent decade, compared with physicochemical properties- and affinity purification-based methods, chemical labeling-, metabolic labeling- or enzymatic reaction-based methods have emerged as powerful tools for the enrichment of cell surface proteins. CCS, cationic colloidal silica [62]; PM, plasma membrane; 2PP, two-phase partition [82,83]; 2PAP, two-phase affinity partitioning [82,83]; FFE: free-flow electrophoresis [76]; FASP: filter aided sample preparation [110]; CSC: cell surface capturing [43]; APEX: engineered ascorbate peroxidase [222]; EXCELL: enzyme-mediated proximity cell labeling [244]

Despite the importance of cell surface proteins, direct analysis of the surfaceome by using the global proteomic approaches has been challenging owing to some technical reasons. As we all know, most of the surface-exposed proteins are embedded with the hydrophobic plasma membrane (PM), as well as many span the lipid bilayer with transmembrane domains, making them harder for the efficient extraction and solubilization. Moreover, transmembrane proteins (TMPs) normally have fewer tryptic cleavage sites, which enhances the difficulty in the separation and identification of tryptic peptides by MS. Besides, cell surface proteins have the characteristic of heterogeneity [21]. Except for the diversity of the transmembrane regions, the compositions and abundance of cell surface proteins and lipids vary with cell types, states, and extracellular environments. Different cell surface proteins possess a wide range and degree of post-translational modifications (PTMs). In addition, cell surface proteins are underrepresented and have an overall low abundance. Cell surface proteins represent about 22% of all proteins in the human genome, of which, only a small fraction has been characterized thus far [22]. The intracellular proteome is often much more abundant than the surfaceome, and direct analysis of the whole proteome always results in low coverage for surfaceome. Thus, prior enrichment of the cell surface fraction is essential for the in-depth analysis of surfaceome.

In general, profiling cell surface proteome is attempted by first enriching cell surface proteins followed by MS analysis. With the technological advancements in MS, a variety of isolation methods have been developed and a brief timeline of the development history of those methods is provided in Figure 1. Based on the separation mechanism, the enrichment methods are mainly divided into three types [23]. The first type is based on their difference in the physicochemical property, such as size, density, charge, and hydrophobicity. The second one is based on the bimolecular affinity interaction with lectin or antibody. And the third type is based on the chemical covalent coupling to free side groups of surface-exposed proteins or carbohydrates, such as primary amines, carboxyl groups, glycosylated side chains. In addition, metabolic labeling and enzymatic reaction-based methods have also been employed to selectively isolate cell surface proteins. In this review, we will provide a comprehensive overview of the enrichment methods for cell surface proteome profiling.

2 | PHYSICOCHEMICAL PROPERTIES-BASED ISOLATION

2.1 | Size- and density-based isolation

Ultracentrifugation and density-gradient centrifugation are the oldest methods for cell surface protein enrichment. Earlier in the 20th century, centrifugation has been proven to be useful to isolate subcellular components according to their differences in size and shape. However, components

with similar sedimentation coefficients cannot be separated. To further increase the resolving power, density-gradient centrifugation was introduced to isolate plant PM system by Hodges and co-workers in 1972 [24]. The density of components plays a secondary role during the separation, which becomes the mainstay of the second stage of subcellular structures purification [25]. In general, cells are first disrupted with the hypotonic buffer in ultracentrifugation or gradient-containing buffer in density-gradient centrifugation. Mild homogenization is followed and damage to organelles is minimal. Next, different subcellular components are isolated with an order of magnitude difference in sedimentation rates for a few hours. In recent years, sucrose gradient centrifugation has been widely used and works effectively for the isolation of PM fraction [26–38].

Ultracentrifugation and density-gradient centrifugation are easy to perform and bio-orthogonal to many other enrichment methods. Thus, they have been widely involved in the enrichment protocols to gain a highly pure cell surface fraction [39–46]. Kasvandik et al. [47] performed cell surface biotinylation method in conjugation with differential centrifugation to profile bovine sperm plasma membrane proteomics. As a result, 338 proteins were commonly identified in all three replicates, of which, TMPs and plasma membrane proteins (PMPs) were highly enriched (on average 35.6 and 40.8%, respectively). Besides, several proteins were first identified on bovine sperm, including CPVL and CPQ. Furthermore, one important advantage of centrifugation-based methods is that they could allow the simultaneous purification for different subcellular components with high throughput and good reproducibility. By taking the advantages, Itzhak et al. [48] developed a global proteomics profiling method based on the sucrose-gradient centrifugation, termed Dynamic Organellar Maps, which could resolve all major organelles with exceptional prediction accuracy (estimated at >92%) and high reproducibility. By combining with the stable isotope labeling by amino acids in cell culture (SILAC) for quantitative analysis, the authors successfully applied the approach to investigate the well-characterized process of epidermal growth factor receptor (EGFR) stimulation. Based on metabolic labeling, this quantification method is mostly suitable for cells in culture. To extend the scope of this method, new workflows by combining with label-free quantification (LFQ) and tandem mass tag (TMT)-based quantification were then developed and applied to mouse neurons and derived a high-resolution quantitative spatial proteome from primary cells [49]. However, the main drawback of centrifugation-based methods is that the organelles, such as mitochondrial and endoplasmic reticulum (ER), could be simultaneously obtained with the PMs due to the similar densities and this could introduce the contaminations to PM fraction [50]. To further improve the PM purity, alkaline carbonate washing was routinely performed for repeated times to reduce non-covalent protein–protein interactions [27,29,30,33,51–53]. Alkaline carbonate buffer has alkaline pH and high ionic strength, which could stimulate the opening of membrane structures and thus release the entrapped contents. As a result, PM fraction with fewer amounts of absorbed cytoplasmic proteins could be obtained [54]. Another drawback of centrifugation-based methods is that they normally require large amounts of starting material and are time-consuming to obtain a crude cell surface fraction, which could lose the composition integrity of cell surface fraction and is a disadvantage for some sensitive structures, such as PTMs.

2.2 | Surface property-based isolation

Cell membranes consist mainly of lipids and proteins and form the boundaries of all cells [55]. The major lipids in bilayer membranes are phospholipids and nearly all cell surface proteins have glycans on the extracellular space, which are all believed to contribute to the intrinsic net electronegative charge of cell surfaces. Besides, cell surface proteins are commonly associated with the hydrophobic cell membrane and most span the lipid bilayer with single or multiple transmembrane domains that are formed by 15–25 non-polar amino acids, which is the major contributor to the hydrophobic characteristic of cell surface proteins. Although the glycosylation on the extracellular space of proteins results in the hydrophilic property of glycopeptides, it is still highly hydrophobic for the TMPs in the cell surface fractions, especially for proteins with multiple transmembrane domains. Overall, taking advantage of the above surface properties, a variety of methods have been introduced to isolate cell surface fractions, including free-flow electrophoresis (FFE), two-phase partition (2PP) system, and several adhesion-based methods. We will give an overview of these enrichment methods.

2.2.1 | Adhesion-based isolation

To reduce both the equipment and the sophistication of the traditional centrifugation-based methods required, adhesion-based methods have been introduced based on the net negative charge of the cell surfaces or the phospholipid structure. Polylysine-coated polyacrylamide and glass beads were successively introduced to covalently bind with negatively charged cell membranes by Jacobson and co-workers in 1977, and the strong binding allowed that cells could be shorn away while the inside-out PM on the cell surface was attached to the beads [56–59]. More recently, polylysine-coated glass plates were employed to isolate quantities of pure PMs for lipid analysis [60]. Cells were first adhered to polylysine-coated glass plates, followed by treatment with the hypotonic buffer and moderate washing buffer to disrupt cells and remove the intracellular organelles, respectively. Finally, the authors analyzed the cholesterol contents of the attached PMs. Later,

Mun et al. [61] further optimized the workflow and successfully applied the method to analyze *N*-glycans on cell surface. Briefly, the attached PM fraction was digested and the glycopeptides were purified by HILIC-SPE, followed by the treatment with peptide *N*-glycosidase (PNGase) F to release the glycans. Finally, MALDI-TOF was used to analyze the glycan profiles. As a result, high-mannose type glycans were more enriched than those obtained by the ultracentrifugation-based method, demonstrating the high extraction efficiency of the cell surface glycans by using the adhesion-based method. Overall, the above polylysine coating-based methods are cost- and time-saving. However, the main drawbacks of the methods are the low (~15%) recovery of PMs and the need for large amounts of starting material for the analysis of PMs. To overcome the limitations, cationic colloidal silica (CCS) method with higher yield was soon introduced by Jacobson and co-workers in 1983. Besides, this method could maintain the trans-bilayer orientation of PMs [62]. For this purpose, intact cells are coated with a dense pellicle of cationic silica particles and anionic polymers, which could greatly enhance the density and stabilization of the PM. Then, ultracentrifugation or density-gradient centrifugation is performed, followed by various elution conditions, like detergent and/or chaotropic agent containing buffer. CCS method has been proven to be useful for the preparation of PM fractions from both the suspended and monolayer cells and even from organ endothelial cells in vivo. Durr et al. [63] first applied this technique to profile the PMPs of endothelial cells (ECs) in vivo and in cell cultures. By combining with multidimensional protein identification technology, 450 proteins in total were identified and 337 proteins have been characterized to some extent according to the current rat database. Importantly, the authors found striking microenvironment-induced molecular differences between ECs in vivo and in vitro, demonstrating the importance of drug-target research in the in vivo condition. To date, a number of cationic nanoparticle pellicles have been synthesized and some are commercially available, like Percoll, Ludox, etc. Choksawangkarn et al. [64] and others have found that nanoparticles with higher densities were more effective to reduce the lysed cytoplasmic components during the centrifugation and enhance the purification efficiency of PMPs. To systematically test the hypothesis, the authors used three cationic nanoparticle pellicles of Fe_3O_4/Al_2O_3 pellicles, Al_2O_3/SiO_2 pellicles, and Ludox to profile the PMPs of multiple myeloma cells, of which, Fe_3O_4/Al_2O_3 pellicle had the highest density. Most of the proteins were commonly identified in three experiments and the best purity (23.6 vs. 18.5%, 16.7%) of PM fraction was enriched by Fe_3O_4/Al_2O_3 pellicle, which confirmed the above hypothesis. However, although CCS has increased the density of PM fraction, it is still not enough to avoid the time-consuming ultracentrifugation or density-gradient centrifugation. Thus, cationic silica-coated magnetic nanoparticles have been synthesized and applied to PM purification, which could allow the method to be simpler and faster [65,66]. Recently, polydopamine (PDA)-coated magnetic nanoparticles were used to enrich the cell surface proteins in HeLa cells, which allowed the identification of 385 PMPs and 1411 non-PMPs. Interestingly, 1316 lipid-raft associated proteins (73.3%) were identified and 85.2% of the identified PMPs were lipid-raft associated proteins, demonstrating that the PDA encapsulation method has excellent performance for the identification of lipid-raft associated PMPs [67]. Raj et al. developed several adhesion-based methods using surface-functionalized superparamagnetic nanoparticles (SPMNP), including oleic acid- and PEGylated phospholipids-coated SPMNP (SPMNP 1.0, SPMNP 2.0, respectively), which allowed the rapid isolation of PM fractions. Very recently, the authors established an integrative approach by combining SPMNP and CCS to isolate PMPs with high purity and high yield [68–71].

Adhesion-based methods have some limitations. The yield of those methods is relatively low and only moderate washing conditions without detergents could be involved to ensure efficient membrane fraction capturing. Moreover, some nanoparticles may also be internalized into cells and introduce the contaminations of cytosolic components.

2.2.2 | Free-flow electrophoresis

Surface properties of bio-particles are associated with their electrophoretic mobility or isoelectric points, which could contribute to the separation of PMs by FFE. FFE, a continuous separation technique, has been proven to be a useful tool in the separation of organelles, cells, protein complexes, etc. [72,73]. Until the 1980s, it was found to be useful for the simultaneous enrichment of PM and the tonoplast fractions in plants [74–76]. At present, there are three basic modes of FFE, including zone electrophoresis, isotachophoresis, isoelectric focusing, of which, free-flow zone electrophoresis has been most widely used for organelle separation. Compared with other organisms, plant PM fractions were mostly separated by FFE [42,77–79]. Recently, to increase the diversity of proteins assigned to the PM, FFE in combination with 2PP was employed to profile the PMPs of *Arabidopsis* seedlings. A population of highly enriched PM vesicles was finally obtained, in which, a total of 1029 proteins were reducibly identified and about 70% were annotated as PMPs. The results showed that FFE has the capacity to purify PM vesicles from crude PM samples with higher resolution [80].

2.2.3 | Two-phase partition

Two-phase partition (2PP) is another method to isolate cell surface fraction according to their difference in surface properties. Earlier in 1958, Albertsson et al. first successfully applied 2PP to separate bio-particles of proteins [81]. For a

2PP system, one or two polymers are mixed in aqueous solution at a given proportion beyond the critical concentration. Bio-particles were then separated due to their different affinities with the two immiscible phases. It should be noted that the concentration and composition of the phase-forming polymers, salts, temperatures, and biospecific ligands could affect partitioning abilities of bio-particles. Until the 1970s, with the development of affinity partitioning [82,83], 2PP has become a powerful separation method to enrich PM fractions [84–88]. To date, the most widely used 2PP system consists of PEG and dextran [89], in which PM fraction tends to enter the PEG-rich upper phase, whereas other subcellular components tend to enter the dextran-rich bottom phase. Besides, Triton X-114 phase partitioning has been a popular choice for the separation of glycosylphosphatidylinositol-anchored membrane proteins [90–92]. With the advantages like, time-saving, low consumption of energy and resources, material-saving, and cost-effectiveness, 2PP has been proven to be useful for the fractionation of a variety of organisms. Moreover, compared with centrifugation-based methods, it is more suitable for small amounts of samples and mammal tissues [89,90,93–98]. Nevertheless, many studies show that 2PP alone is not sufficient to eliminate contaminating membranes, and some improvements have been employed to further enhance the purity of PM fractions. First, affinity ligands, mostly lectins, like wheat germ agglutinin (WGA), concanavalin A, could be conjugated to one of the phase polymers, termed two-phase affinity partitioning (2PAP) [39,94,96]. Schindler et al. [94] found that highly enriched PM fraction could be obtained by combining the PEG-enriched phase with a subsequent 2PAP system based on WGA, and 34–42% of the identified proteins were annotated as PMPs. Second, prior enrichment of PM fractions by sucrose density centrifugation could also help increase the specificity of the 2PP method [39,98,99]. Khanna et al. [39] found that an integrative strategy by combining density gradient centrifugation with 2PAP could help obtain a robust PM fraction, while 2PAP alone could not completely remove contaminating ER and mitochondrial membrane proteins. By using this strategy, a total of 432 proteins were successfully identified using multidimensional protein identification technology, of which, 37% were integral membrane proteins from all compartments. Third, the purity of the PM containing phase could be further enhanced by harsh washes with high salt and high pH buffers [96,97]. In this way, non-covalent protein–protein interactions could be destroyed, which results in a reduced number of non-specific proteins in PM fractions. Additionally, the high-pH treatment also has the capacity to remove soluble proteins embedded in sealed membrane compartments. Apart from the above improvements, repeated rounds of 2PP also help to increase purity but inevitably decrease the final yield of PM fraction [80,95,99]. Despite the limited specificity, 2PP systems are sensitive to salt, temperature, and several other factors, which thus require proper and careful handling at defined conditions for better performance. Since only about 70% of PM fractions could be retained in the upper phase, the apparent loss of membrane material is inevitable. In addition, the exposure of membranes to polymers, like PEG, may result in incompatibility with subsequent experiments.

3 | AFFINITY-BASED ISOLATION

The composition of cell surface proteins varies with cell types, developmental stages, and environments. Aberrant composition, either an alteration in cell surface protein expression or the glycosylation profile on cell surface proteins could lead to cellular dysfunction and cause diseases [100]. Antibody-affinity purification is useful for known cell surface protein profiling, which enables multiple-sample handling with high sensitivity and specificity. However, the method is normally expensive and antibodies with high specificity are required. Furthermore, only known cell surface proteins are allowed to be analyzed and thus it is not suitable for in-depth cell surface protein profiling at present.

Compared with antibody-affinity purification, lectin-affinity purification has been more widely used to enrich cell surface proteins, especially cell surface glycoproteins. Most cell surface proteins carry sugar residues on extracellular space. Glycosylation has been intimately associated with many diseases, such as cancer, and surface glycans or glycoproteins could serve as effective biomarkers for cancer diagnosis, such as CA125 for ovarian cancer, CA19-9 for gastrointestinal and pancreatic cancer. However, the low abundance, high heterogeneity, and dynamic range of glycosylation make the global analysis of cell surface glycoproteins very challenging. Lectin-affinity purification has been used to profile glycoproteins with specific types of glycan structures. One certain type of lectin can selectively recognize particular carbohydrate residues on proteins: concanavalin A, which binds to mannose, is one most commonly used lectin [90,101–108]; WGA [90,102,103,105,106], which binds to sialic acid, as well as *N*-acetylglucosamine; Jacalin [90,103], which binds to *O*-glycans for its recognition of galactosyl (β-1,3) *N*-acetyl-galactosamine, lactose, and galactose; agglutinin RCA_{120} [105,106,109], which captures galactose modified at the 3-0 position, as well as terminal galactose. Multi-lectin enrichment method has been preferentially performed to map more comprehensive glycoproteomes [90,103,105,106]. Considering the inherent hydrophobic property of membrane proteins, Mann group developed a highly efficient method, termed N-glyco-FASP, by integrating filter aided sample preparation (FASP) [110], multi-lectin affinity purification, and high-accuracy MS characterization for in-depth *N*-glycoproteome mapping. Glycosylated peptides were selectively enriched by binding

to lectins on the top of filters, which allowed the identification of 6367 *N*-glycosylation sites on 2352 proteins from four mouse tissues and blood plasmas [105]. Later, to enable a precise quantitative comparison of multiple samples, a super-SILAC approach was combined to profile the cell surface proteomics of diffuse large B-cell lymphoma subtypes. A total of 2383 glycosylated sites on 1321 protein groups were mapped, which enabled efficient classification of closely related cancer subtypes. Eventually, the authors found that characterizing tumors on the protein- and PTM-level could help classify cancer types and reveal cancer-specific mechanistic changes. However, neither single nor several types of lectins could cover all the glycan structures, which limits the wide applications of lectin-affinity purification method in the analysis of cell surface glycoproteome.

4 | CHEMICAL LABELING-BASED ISOLATION

4.1 | Coupling with primary amines

Lysine is one of the most abundant amino acid residues in proteins (~6% of all residues [111]) and the ε-amine of lysine is intrinsically nucleophilic and is often exposed on the surface of proteins [112], which make it a potentially attractive candidate of chemical labeling target for unbiased cell surface protein profiling. Thus, cell surface biotinylation method, by selectively labeling surface-exposed primary amines of lysine and *N*-terminus has been a powerful method for the enrichment of cell surface proteins. For this purpose, cell surface-exposed proteins are first covalently labeled by a biotinylation reagent, followed by subsequent protein- or peptide-level enrichment. This method, first described by Hurley and co-workers in 1985 [113], utilized a chemical reagent, Sulfo-NHS-biotin, to selectively label surface-exposed proteins of bovine leukocytes. In recent years, a variety of commercially available or laboratory-developed reagents have been designed and applied for the analysis of cell surface proteins [114–116]. Typically, biotinylation reagents consist of three essential groups: (i) a reactive moiety for the covalent binding of biotin to the proteins, *N*-hydroxysuccinimide ester (NHS) is commonly employed; (ii) a linker between the biotin and reactive moiety, which is associated with the reagent's water solubility, membrane permeability, the ability to cleave the biotin group off the labeled protein, etc. Most importantly, it should provide sufficient space allowing the labeled protein to be captured by affinity beads; (iii) a group, mostly a biotin moiety, sometimes a fluorescein tag, alkynyl group, sulfhydryl moiety, etc., which could enable the detection and/or isolation of labeled proteins [1,2,115]. For steric hindrance reasons, Sulfo-NHS-biotin was soon replaced by Sulfo-NHS-LC-biotin, which, with a long-chain spacer, has been a popular reagent on the market for many years. However, several studies found that the hydrophobic long-chain could result in the problems of agglutination, precipitation, and membrane-permeability, which limits the widespread use of the reagent for the analysis of cell surface proteins [117]. More recently, another commercially available reagent, Sulfo-NHS-SS-biotin, with a disulfide linkage, is cell membrane-impermeable, cleavable, and highly soluble in aqueous buffers, making it the most popular reagent for studying cell surface proteins at present [7,23,114]. Besides, a commercial cell surface protein isolation kit based on the reagent has been developed and widely used for cell surface protein profiling [118–123]. With the commercial cell surface protein isolation kit, PosthumaDeBoer et al. [123] performed a comprehensive analysis of cell surface proteomics by combining with gel fractionation from five osteosarcoma (OS) cell lines and three human primary osteoblasts (hp-OBs). As a result, a total of 2841 proteins were quantified, of which, 156 cell surface-annotated proteins were found significantly upregulated in OS cells versus hp-OBs. With follow-up verifications, the authors found EPHA2, a highly upregulated protein, potentially acted as a receptor for the intracellular delivery of targeted vectors in OS cells. Very recently, Elia presented a detailed protocol with the reagent of Sulfo-NHS-SS-biotin for the profiling of cell surface proteins by western blotting analysis [7].

At present, cell surface biotinylation method has been one of the most frequently used methods for cell surface proteome profiling. Several advantages of the method can be numbered. First, cell surface biotinylation method is unbiased and easy to perform. Efficient surface labeling could be completed in one-step and followed by treatment with blocking buffers, such as glycine- or tris-containing buffer. Besides, the information of glycan structures could be kept. It is suitable for almost all organisms and has been successfully applied to the in vivo biotinylation labeling for cell surface protein profiling [119,124–129]. Early in 2003, Thomas-Crusells et al. [130] applied the method to acute brain slices of adult rodents. By combining with immunoblotting analysis, the method allowed studying the protein trafficking mechanism under more physiological conditions, which was further validated by the two previously well-characterized cell surface receptors, glutamate receptor subunit A (GluR A), and transferrin receptor (TfR). More recently, Smolders et al. [125] presented an effective plasma membrane proteomics approach for small tissue samples by integrating cell surface biotinylation method, affinity purification, tube-gel digestion, and shotgun proteomic analysis. A total of 1698 proteins were identified from five brain acute tissue slices, of which, 1011 (62.2%) proteins were annotated as PM-associated proteins. Rybak et al. [129] first extended the cell surface biotinylation method to in vivo biotinylation labeling based on the terminal perfusion of rodents in normal organs and solid tumors. In

addition, according to previous studies, cell surface biotinylation method has the ability to identify more cell surface proteins than most other enrichment methods [5,22,23,119, 131–134]. Recently, Hörmann et al. [23] compared cell surface biotinylation, cell surface aminooxy-biotinylation, and cell surface capturing (CSC) method for cell surface proteome profiling. Not surprisingly, cell surface biotinylation method identified the largest number of absolute PMPs and cell surface aminooxy-biotinylation resulted in the highest purity of PMPs. Compared with the common SDS elution strategy, a competitive biotin elution strategy achieved higher purity and better reproducibility. Except for above advantages, cell surface biotinylation method allows unambiguous verification of the cell surface localization of the identified protein via the assigned MS/MS signal by the introduction of a biotin moiety, which could further help topology prediction of TMPs. Langó et al. [135] applied cell surface biotinylation method to profile the TMP topology information from three different cell lines based on peptide-level enrichment. As a result, 730 (47%) labeled sites in 198 (38%) TMPs, 593 (38%) labeled sites in 212 (40%) intracellular, and 250 (16%) labeled sites in 114 (22%) extracellular proteins were identified with a strict filter from HL60, K562, and red blood cells, respectively, which contributes to a more accurate topology prediction of the human transmembrane proteome. In the future, the high-throughput cell surface biotinylation method could be performed to enhance the large scale topology prediction of human TMPs.

However, several drawbacks have been reported for cell surface biotinylation and improvements have been developed to optimize the protocol [8,23,121,131,136]. A main drawback of the method is that cytoplasmic contaminations are always observed, which compromises the specificity for cell surface proteins. Commonly, considering the high affinity of the biotin-avidin system ($K_d \sim 10^{-15}$ M), purity could be enhanced by harsh washes with high pH-, high salt-, strong detergent, and chaotropic buffers during the affinity purification [8,22,47,131,137–142]. By integrating selective biotinylation method, affinity enrichment, in-gel digestion, and MS proteomics profiling, Zhao et al. [142] optimized the protocol of cell surface biotinylation method, termed biotin directed affinity purification (BDAP). Harsh washes with high-salt and high-pH buffers were introduced to reduce PM-associated cytosolic proteins. Finally, in-depth integral plasma membrane proteome profiling, including a total of 526 (67.3%) integral PMPs, was obtained from a human lung cancer cell line. Besides, by combining with other isolation methods, the purity of PMPs could be further enhanced [34,47,131,139]. Ultracentrifugation has been often performed before the affinity enrichment to first get a crude PM fraction. In addition, the labeling reagents are also crucial for high purity. Typically, labeling reagents have the tendency to cross the PM. The disulfide linkage, cleavable in the reducing cytoplasmic environment, helps selectively label the cell surface proteins and elute the cell surface fraction from the affinity beads, which could further enhance the purity of PM fraction. In addition, although cell surface biotinylation method allows a smaller number of starting cells than centrifugation-based methods and CSC technology, the requirement (typically $\sim 10^7$ cells per experiment) is still not always available for some cells, such as stem cells and primary cells. More recently, our group optimized the protocol of cell surface biotinylation method, mainly the elution procedure, and more cell surface proteins were identified from a reduced amount of starting material ($\sim 10^6$ cells per experiment) [143]. With the optimized protocol, a total of 2932 proteins were identified form HeLa cell lines, including 1401 (47.8%) high-confidence cell surface-associated proteins, 91 CD (clusters of differentiation) antigens, and 742 TMPs. By using a special pipet tip, the in-depth cell surface proteome analysis could be achieved with high sensitivity. About 600 cell surface-associated proteins could be identified from only 1×10^5 Hela cells, and 2055 cell surface-associated proteins were identified from 4×10^6 cells [143]. Except for the above drawbacks, cell surface biotinylation method may not be applicable for cell surface proteins with no accessible primary amine on the extracellular region [144] and thus complementary strategies could help gain a more comprehensive mapping of cell surface proteins. Besides, another drawback of the method is that biotinylation of surface lysine side chains could block subsequent tryptic digestion during sample preparation [145].

Very recently, engineering individual cell surfaces with dense inorganic or organic shells has been proven to be a promising approach for protecting cells from hostile environments [146–152]. Functional polymers have been successfully introduced onto the cell membrane mainly by "depositing-to" approach, "growing-from" approach, or interfacial reactions, which holds the potential to isolate cell membrane proteins in the future [153]. Wu et al. [154] selectively isolated the cell membrane proteins through cell surface-initiated atom transfer radical polymerization by "grafting from" approach. This new approach involved the cytocompatible modification of thermo-responsive polymers onto cell surface-exposed primary amines, which enabled easy separation and enrichment of cell membrane proteins by thermo-precipitation. As a result, totally 1825 proteins, including 1036 (71.7%) specific membrane proteins, were identified from *E. coli.* However, the current protocol involved extensive sample-handling steps, including some harsh conditions in living cells, which need to be further improved. Overall, surface grafting strategy has the potential to be a powerful technique for cell surface proteome profiling in the future.

4.2 | Coupling with carboxyl groups

Surface exposed-carboxyl groups on aspartic acid (Asp), glutamic acid (Glu), and protein C-termini also could be utilized to isolate cell surface proteins. Although the frequencies of Asp and Glu residues, especially on the extracellular TMPs, are larger than that of Lys residues according to the recent databank entries, surface-exposed carboxyl groups have been rarely utilized for the cell surface proteome profiling compared with surface-exposed carbohydrates or primary amines [50,155]. For this purpose, carboxyl groups should be firstly activated by water-soluble carbodiimides to form active esters, followed by covalently reacting with amine-derivative of biotins. To gain a comprehensive understanding of the surface-exposed outer membrane proteome, Voss et al. [155] performed four complementary biochemical and biophysical enrichment methods. As a result, a total of 39 high-confidence cell surface and outer membrane proteins were identified from *Helicobacter pylori*, of which, many proteins were previous known or predicted to be localized on the cell surface. Biotinylation strategies using amine-reactive and carboxyl-reactive reagents were both employed and similar surface-exposed proteins were identified by both strategies. Out of 32 proteins identified by carboxyl-reactive biotinylation strategy, 27 proteins were also identified by the amine-reactive biotinylation strategy. Very recently, Özkan Küçük and co-workers carefully optimized carboxyl-reactive biotinylation protocol and presented the first example of targeting the carboxyl groups on the cell surface for mammalian cell surface proteome profiling. Out of 219 identified proteins from HeLa S3 cells, 82% were annotated as PMPs, demonstrating that carboxyl-reactive biotinylation method was much more selective and efficient than amine-reactive biotinylation method (31%, 530). However, the carboxyl-reactive biotinylation method requires more steps, including the activation of carboxyl groups, which needs to be further optimized in the future. Besides, a combination of carboxyl- and amine-reactive biotinylation methods is recommended for more comprehensive cell surface proteomics studies. For this purpose, biotinylated proteins obtained from both methods could be simply combined for subsequent affinity purification.

4.3 | Coupling with glycan chains

Protein glycosylation has long been recognized as a very common and important posttranslational modification [156], which is essential for cell survival and has crucial roles in a wide variety of extracellular activities [1,157]. Besides, it is mainly in the extracellular region of proteins. In vertebrates, all cell surfaces are decorated with a dense layer of glycans [158] and nearly all cell surface proteins (~90%) are glycosylated [157], making glycan chains of surface-exposed proteins attractive "handles" for selectively capturing and analyzing cell surface-exposed proteins. At present, there are three main strategies for cell surface glycoprotein enrichment: lectin-affinity purification, chemical glycocapture strategy, and metabolic labeling. In this part, we will provide a detailed overview of chemical glycocapture strategy for glycoprotein enrichment.

Low concentrations of periodate could selectively oxidize externally exposed cis-diols of sialic acids to aldehydes, which was first described 40 years ago by Carl and co-workers [159]. Sialic acids are a family of negatively charged nine-carbon monosaccharides that often reside at the outermost ends of glycan chains. Till 2003, one covalent capturing procedure based on hydrazide chemistry was introduced to directly target the above-generated aldehydes by Zhang and co-workers [156], which allowed the selective enrichment of *N*-linked glycoproteins in a complex sample. This approach has been proven to be a robust and highly effective strategy for glycoprotein profiling of blood serum and cellular samples. Since most cell surface and secreted proteins are known or predicted to be glycosylated, this approach was found to be useful for the analysis of cell surface glycoproteins [22,90, 100,160]. Briefly, living cells are treated with periodate and then lysed. Next, protein-level or peptide-level *N*-glycoprotein enrichment using hydrazide chemistry is performed followed by subsequent stable isotopic labeling and specific releasing with PNGase F. Finally, the glycoproteins could be identified, sequenced, and quantified simultaneously. Sun et al. [160] developed an optimized protein-level enrichment strategy by integrating the information of non-glycosylated and glycosylated peptides from two human cell lines. As a result, in total 341 glycoproteins were identified with 82.4% specificity for cell membrane proteins and 33 glycoproteins were quantified with significant expression changes between the two cell lines. Recently, Fang et al. [161] also developed a novel PM enrichment strategy based on hydrazide chemistry, termed glycan moiety-directed PMPs enrichment (GMDPE) strategy. Glycosylated and non-glycosylated PMPs were isolated by the raft-binding effect of the phospholipid bilayer, followed by covalently capturing the surface glycan moieties using hydrazide chemistry. As a result, 772 PMPs were identified from HeLa cells, which increased by 4.5 times compared with the cell surface biotinylation method. By integrating enzymatic and chemical reactions with MS-based proteomics, Sun et al. [162] recently developed a novel method for global and site-specific analysis of *N*-glycoproteins. After removing the sialic acids, living cells were treated with galactose oxidase (GAO) and horseradish peroxidase (HRP) instead of the periodate to specifically oxidize the Gal/GalNAc on cell surface glycoproteins to aldehyde groups. Next, methoxylamine instead of PNGase F was added to elute the enriched glycopeptides for the follow-up glycopeptides analysis. With the new method, on average 953 *N*-glycosylation sites on 393 surface glycoproteins per experiment were identified from

MCF7 cells in triplicate biological experiments, demonstrating that the high efficiency and selectivity of the method for the global and site-specific cell surface glycoproteome profiling.

To further enhance the specificity, two-step covalent capturing strategies for the cell surface-exposed glycoproteins were successively developed by Zeng et al. [163] and Bernd et al. [43] in 2009. Briefly, the aldehydes generated by periodate oxidation on cell surface glycoproteins were selectively probed by membrane-impermeable reagents based on the aniline-catalyzed oxime ligation [164] or hydrazide chemistry in living cells [165] and followed by subsequent affinity purification, termed cell surface aminooxy-biotinylation, and CSC, respectively. Both strategies could enable the comprehensive and quantitative analysis of the cell surface glycoprotein landscape with very high specificity. Weekes et al. [166] applied cell surface aminooxy-biotinylation to profile the differential expressions of cell surface glycoproteins on gp96-deficient versus gp96-reconstituted murine pre-B cells. By integrating an off-line fractionation strategy and SILAC labeling, 1271 proteins were quantified, of which, 564 proteins were annotated as PMPs. Interestingly, four members of the extended LDL receptor family were included in the list of the 29 down-regulated proteins in gp96-deficient cells (P value < 0.05). With follow-up validation with flow cytometry, the authors found that optimal cell surface expression of the LDL receptor family is regulated by gp96. Recently, Kalxdorf et al. [167] optimized the protocol of aniline-catalyzed cell surface aminooxy-biotinylation. By integrating protein-level enrichment and improved MS instrumentation, in-depth cell surface *N*-glycoproteome analysis was obtained from 15 standard laboratory human cell lines and three primary lymphocytic cell types. On average, 835 PM-associated proteins were identified with high reproducibility, including 322 PM receptors and transporters. Importantly, by combination with isobaric mass tag-based chemical labeling strategy, cell surface proteome dynamic changes during the differentiation of the monocytic suspension cell line THP-1 into macrophage-like adherent cells were monitored with a depth of more than 1000 cell-surface proteins in a single experiment. Except for the cell surface aminooxy-biotinylation method, CSC technology has been a more popular tool for cell surface glycoprotein analysis in the past decade. Bernd et al. [43] first developed the CSC technology to enable the specific view of cell surface protein landscape in qualitative- and quantitative-level. Bernd group later employed the CSC technology in conjugation with LFQ proteomics to profile the perturbation-induced surface proteome changes in the *D. melanogaster* Kc167 cell line [168]. To further increase the sensitivity and cover more captured peptides, Bernd group optimized the original CSC technology and established three complementary CSC technology variants, termed Cys-Glyco-CSC, Glyco-CSC, and Lys-CSC [169]. Very recently, Leung et al. [170] performed a multi-proteomics analysis of the DNA methylome, the transcriptome, and the cell-surface proteome to characterize the effects of Azacitidine (AZA) treatment on four AML cell lines in different stages of differentiation. To date, CSC technology has been applied to the pluripotent stem cells [171–173], kidney collecting duct cells [144], primary adipocytes [174], neural stem cells [175], human malignant lymphocyte cell lines [176], human colon cancer cells [177], etc. In 2015, to get a comprehensive mapping of cell surface proteome atlas, Bernd group applied the CSC technology to 41 human and 31 mouse cell types and generated a surfaceome database of cell surface protein atlas containing 1492 human and 1296 mouse high-confidence cell surface glycoproteins. In addition, with the identified *N*-glycosylated sites, the authors proposed refined topology models for 51 human and 39 mouse proteins [178]. Afterword, Bernd group build an open-source tool, termed Protter, for the visualization of proteoforms and interactive integration of annotated and predicted protein topology together with experimental proteomic data [179]. More recently, using the high-confidence cell surface glycoproteins from cell surface protein atlas as a training set, Bernd group developed a computational cell surface protein prediction tool, termed SURFY, to complement the experimental resource. As a result, in total 2886 cell surface proteins from human proteome across all human cell types and developmental stages were predicted with a 5% FDR [4]. In recent years, based on the bifunctional reagents, Bernd group specially designed trifunctional reagents, TRICEPS [180,181] and HATRIC [182], by introducing a functional NHS group to conjugate the reagent with a free primary amino group-containing ligand. The authors then applied the trifunctional reagents to discover the corresponding cell surface orphan *N*-glycoprotein-receptors, termed ligand-based receptor capture (LRC) technology. At present, the reagents have emerged as highly selective and efficient tools for the direct identification of ligand-receptor interactions. In the future, more trifunctional reagents will be designed and applied to the analysis of cell surface receptors, enzymes, and so on [183].

Classical CSC technology enables highly specific enrichment of cell surface *N*-glycoproteins and topology analysis with the information of glycosylated sites obtained by direct identification of surface biotinylated *N*-glycopeptides. While, the information of non-glycopeptides for the cell surface glycoproteins is missed that results in the cost of sensitivity; on the other hand, the extensive sample-handling steps involved in the workflow, including the two-step labeling, hypotonic buffer treatment, also contribute to the sample loss and the insufficient sensitivity. Commonly, CSC technology requires a large amount of starting cells (10^7 to 10^8 cells per experiment) and identifies relatively small numbers of cell surface proteins [171,184]. Thus, it is unsuitable for a very small amount of cell lines. More

recently, Bernd group miniaturized and automated the classical CSC technology to reduce sample loss during manual processing, termed autoCSC. By physical confinement of the reaction space and automation of the biotin-streptavidin system, enhanced sensitivity and quantitative reproducibility were allowed compared with the manual workflow. The authors first applied autoCSC to 11 commonly used cancer cell lines and found that autoCSC could reliably subclassify cell types based on the surfaceome. Furthermore, by combining with target proteomics of data-independent acquisition strategy, the authors applied the autoCSC to de novo map and quantitatively compared the surfaceomes of nine consecutive stages of B cell populations with a maximum of 1×10^6 cells per sample. Population-specific surfaceome maps of developing mouse B cells were created with a depth of totally 248 unique glycosylation sites in 147 protein groups. With follow-up investigations, the immature B stage was further split into three subpopulations with different maturities by cell surface *N*-glycoproteins CD20 and CD180 [185]. Except for the requirement of a large number of starting cells, another drawback of the CSC technology is that protein quantification may be affected by alterations in *N*-glycosylation status. Besides, protein quantification could be challenging by relying on a single modified peptide, and that unique peptide identified from one protein do not always follow the same quantitative trend.

5 | METABOLIC LABELING

In the recent two decades, metabolic labeling with click chemistry has opened a new avenue for the enrichment of cell surface glycoproteins [157]. For this purpose, unnatural sugar analogs containing bio-orthogonal groups are fed to living cells and metabolically engineer cell surface glycans through the biosynthetic machinery. Subsequently, membrane-impermeable probes are introduced to covalently react with the sugar analogs using click chemistry in living cells. Then, the cells are lysed and exploited for highly efficient purification of cell surface glycoproteins. Bertozzi and co-workers first engineered cell surfaces by a metabolic labeling approach and subsequently performed many pioneering works by using unnatural sugar analogs to label glycoproteins [186–192]. To date, the global and quantitative analysis of glycosylated proteins, peptides, and sites have been achieved using this strategy [193,194]. A variety of sugar analogs have been developed for metabolic labeling, including *N*-azidoacetylgalactosamine (GalNAz), *N*-azidoacetylglucosamine (GlcNAz), *N*-azidoacetylmannosamine (ManNAz) and fucose analogs. And azidosugars have been most widely used owing to the small size of azido group, essential absence in the biological system, good orthogonality with cellular functional groups, and kinetic stability under physiological conditions [194,195]. The copper-free click reaction is quick, specific, cytotoxic heavy metal ions-free, and occurs under physiological conditions, making it ideal for metabolically tagging surface sialoglycoproteins in living cells [196]. Since surface glycoproteins are dynamic for living cells in response to the ever-changing extracellular environment, metabolic labeling method, which allows selectively labeling cell surface glycoproteins under more physiological conditions, has the potential to monitor the dynamic cell surface glycoproteomics. In recent years, Wu group developed many novel strategies for the global and temporal profiling of surface glycoproteins based on metabolic labeling and copper-free click chemistry [157,196–198]. Recently, Wu group designed a novel strategy to systematically analyze surface glycoprotein dynamics and measure their half-lives by integrating pulse-chase labeling, selective enrichment of surface glycoproteins, and six-plexed TMT quantitative proteomics method. Moreover, since only newly synthesized cellular glycoproteins could be metabolically labeled with the sugar analogs, metabolic labeling has the possibility to enable the simultaneous analysis of surface glycoproteome and secretome in the presence of serum or other protein-containing cell culture medium supplements [145,196]. Based on previously developed click sugars-based secretome protein enrichment method (SPECS) [199], Herber et al. [145] recently developed a novel method, which enabled the simultaneous global analysis of cell surface glycoproteome and correlative secretome in one experiment, termed "surface-spanning protein enrichment with click sugars" (SUSPECS). By combination with LFQ proteomics, a total of 471 transmembrane glycoproteins were consistently quantified, of which, 21 transmembrane glycoproteins were regulated with the treatment of BACE1 inhibitor. However, metabolic labeling has slow reaction kinetics and needs a relatively long time to realize efficient labeling of cell surface glycoproteins. Moreover, there are some cases where proteins have very long half-lives, or the newly synthesized glycoproteins could incorporate their own glycan metabolic precursor, which could result in the non-efficient labeling. Besides, the click chemistry, even the copper-free click chemistry is toxic for living cells [1]. Except for the above drawbacks, metabolic labeling has been mostly applied in cultured cells and its applications to tissue or clinical samples are restricted at present. Efforts have been employed to extend the application scopes [192].

6 | ENZYMATIC-BASED ISOLATION

Surface-exposed proteins are highly accessible to exogenous treatment in living cells. Taking advantage of the properties of enzymes, like proteolysis, biocatalytic oxidation, enzymatic-based isolation has emerged as an important

tool for cell surface protein profiling. This can be further divided into enzymatic shaving method and enzymatic coupling method at present. Enzymatic shaving method, using proteolytic enzymes to 'shave' surface-exposed proteins on intact cells, has emerged as a well-established method to enrich surface-exposed proteins and also could help topology prediction of TMPs. In 2006, Rodríguez-Ortega et al. [200] first established the method to identify the surface-exposed proteins of bacterial cells with the trypsin and proteinase K. As a result, 72 proteins were identified from the M1_SF370 stain, of which, 68 (94%) proteins were predicted to be surface-associated proteins, including 12 cell wall-anchored proteins, 11 lipoproteins, 37 TMPs, and eight secreted proteins. Enzymatic shaving method is simple, time-saving, and has been widely applied to the cell surface protein profiling of gram-positive bacteria, such as *Streptococcus pyogenes* [35], *Listeria monocytogenes* [201], *Bifidobacterium* [202], *Enterococcus faecalis* [203], *Staphylococcus aureus* [204–208]; gram-negative bacterium, such as *Capnocytophaga canimorsus* [209]; parasites, such as *Borrelia* species [132]; and eukaryotes cells [210], such as *Aspergillus fumigatus* [211], *Rhodopirellula baltica* [212]. Trypsin-shaving approach has been most often employed [5,132,205,207,208,210,211,213–215] and protease K-shaving approach [155,203,212] takes the second place. In addition, a combination of trypsin-shaving and protease K-shaving approaches also has been employed for in-depth cell surface proteome profiling [200,216]. However, one important drawback of enzymatic shaving method is that cytoplasmic contaminations from the cell disruption are often involved during the proteolytic digestion of surface-exposed proteins [205]. Many efforts have been employed to optimize the enrichment protocols, including using immobilized enzymes instead of soluble enzymes [131,217], which could avoid penetrating into the cell wall. Additionally, a false-positive control group could be added [5,213,218], in which intact cells are incubated without enzymes under similar conditions [205,217]. Cordwell and co-workers have reported several improvements to reduce cytoplasmic contaminations. In 2014, they developed a novel probability scoring approach for enzymatic shaving proteomics. Trypsin-shaving approach using a false positive control was performed to compare the surfaceomes of three strains, including methicillin-resistant *S. aureus* COL strain (COL) and two oxacillin-adapted *S. aureus* COL strains (APT, grown in tryptic soy broth or tryptic soy broth/NaCl + oxacillin, respectively). As a result, 150 surface-exposed proteins were identified after filtering thresholds by the new scoring algorithm from the three strains and a smaller number of surface-exposed proteins were identified in both APT strains. Quantitative RT-PCR analyses and quantitative isobaric tags for relative and absolute quantitation on the whole proteome were then performed, which indicated the changes of the surface architecture of *S. aureus* in response to antibiotic adaptation [208]. In addition, just as cell surface biotinylation method, enzymatic shaving method also targets all surface-exposed proteins, including the extracellular secreted proteins, which may be not true cell surface proteins. Considering the larger size of enzymes than the biotinylation reagents, steric hindrance is much larger for enzymes to access TMPs [206] and thus TMPs tend to be identified by the biotinylation approach. Multiple complementary enrichment methods, such as a combination of enzymatic shaving and cell surface biotinylation methods, are recommended to get a more comprehensive analysis of cell surface proteins [5,132,155,201,209].

Recently, the enzymatic-coupling method has emerged as a powerful approach to study protein behavior and subcellular proteomics in living cells. Generally, enzymes that produce reactive molecules are introduced to covalently label neighboring proteins. Subsequently, the labeled proteins could be isolated via conventional affinity purification and identified by MS. It enables the detection of low-affinity and/or transient protein–protein interactions under physiological conditions [219]. In the recent decade, multiple methods have been developed, including engineered ascorbate peroxidase-based methods (APEX [220–222], APEX2 [223–228]), promiscuous biotin ligase-based methods (BioID [229–231], BioID2 [232,233], BASU [234], TurboID [235], miniTurbo [235]), HRP-based methods (EMARS [236–240], SPPLAT [241,242]), etc. Among them, EMARS and SPPLAT have been mainly employed to study protein–protein interactions on the cell surface. Other methods have been mostly applied to study membrane-enclosed compartments and applications for global cell surface proteome profiling have been rarely reported to date. In 2016, Ting group [226] extended the APEX platform to the proteomics analysis of the "open" subcellular region, neuronal synaptic cleft, where APEX2 was replaced with HRP. By a "ratiometric" APEX-tagging strategy, two independent proteomic lists for excitatory and inhibitory synaptic cleft were generated, which mapped 69 and 46% of well-established excitatory proteins and inhibitory cleft-resident proteins, demonstrating the good sensitivity of the approach. Recently, Conlan et al. [243] developed an in planta BioID system to rapidly determine protein interactions in living plant tissue. In combination with AvrPto, PM-associated immunity proteins were isolated and identified by MS. After excluding the proteins identified by control groups, five proteins were identified to be AvrPto proximal plant proteins. Further verification found that these five proteins mainly have the function on plant immune function and growth. Very recently, Chen et al. [244] evolved a "promiscuous" *Staphylococcus aureus* transpeptidase sortase A variant (mgSrtA) to monitor cell–cell interactions by flow cytometric analysis, histograms of biotin staining, and fluorescent microscopy, termed enzyme-mediated proximity cell labeling (EXCELL) strategy. In the future, by combining

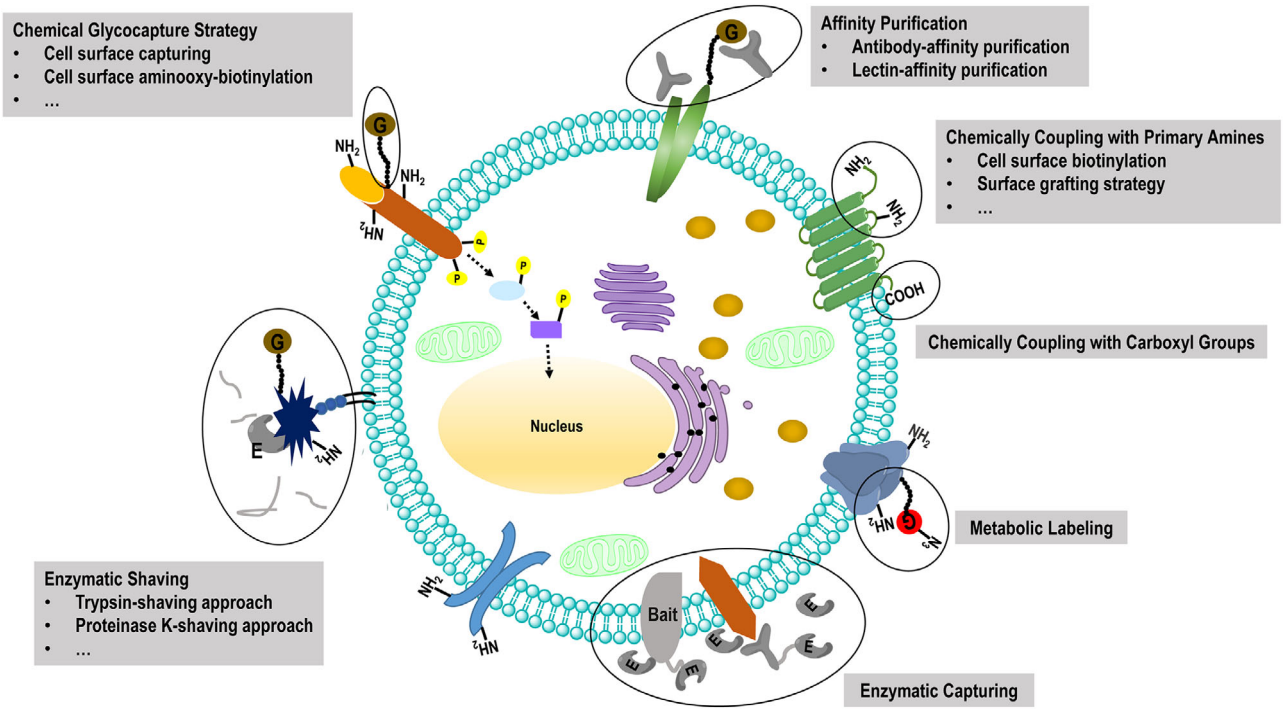

FIGURE 2 Schematic overview of different chemical lab eling-, metabolic labeling- and enzymatic reaction-based methods for the enrichment of cell surface proteins

with proteomic profiling, the method holds the potential to detect unknown cell–cell interactions. However, most available enzymatic capturing methods need to firstly target the evolved enzyme to a specific protein or subcellular compartment by genetic fusion. Currently, we are using exogenous enzymes to establish a universal cell surface protein isolation method, which is easy-operational, time-saving, and has good specificity for cell surface proteins. Most importantly, it has rapid labeling kinetics to capture the dynamic surfaceomes responding to extracellular stimuli.

7 | SUMMARY AND PERSPECTIVES

Cell surface proteome profiling has been of fundamental importance for understanding biological systems and serves for drug target screening, vaccine development, biomarker discovery, etc. With the development of MS techniques, significant advances have been achieved for cell surface proteome profiling. In this review, we mainly focus on the methods for the enrichment of cell surface proteins. In the recent decade, compared with physicochemical properties and affinity purification-based methods, methods based on chemical labeling, metabolic labeling, and enzymatic reactions have emerged as powerful tools for the enrichment of cell surface proteins and an overview of those methods is given in Figure 2. Currently, for mammals, cell surface biotinylation method and CSC technology have been commonly involved. For parasite and bacterium, especially gram-positive bacterium, enzymatic shaving method and cell surface biotinylation method have been often employed. For plants, the global analysis of cell surface proteome has been rarely reported, and ultracentrifugation and density-gradient centrifugation remain popular choices. Multiple complementary biochemical and biophysical methods have been often employed to gain an in-depth analysis of the cell surface proteome.

Although each method could be particularly valuable for varying experimental goals, several common drawbacks need to be further optimized in the future. In general, specificity and sensitivity are two important factors that we often need to consider. At present, most available methods have the drawback of cytoplasmic contaminations in varying degrees and the verification of surface localization for the identified proteins using database is necessary, which is a disadvantage to find unknown cell surface proteins. Several strategies have been developed to reduce the cytoplasmic contaminations. Ultracentrifugation and density-gradient centrifugation that are biorthogonal to many methods have been commonly employed in conjugation with other methods. Besides, harsh washes with high pH-, high salt-, strong detergents-, and chaotropic buffers have been often employed to reduce cell surface-associated cytosolic proteins. Except for the poor specificity, most current methods have relatively low sensitivity and need a large amount of starting cells (10^7 to 10^8 cells per experiment), which are often not available for primary cells, stem cells, etc. Thus, optimization should be further performed for current methods to enhance the sensitivity and specificity of the methods. On the other hand, novel

methods allowing unbiased identification of cell surface proteins with good sensitivity and specificity also need to be established. Except for the sensitivity and specificity, methods with rapid labeling kinetics have the potential to help us get a more comprehensive understanding of the temporal changes on cell surfaces. The compositions and topologies of cell surface proteins in living cells are dynamic to adapt to their environment. Actually, the research trends for cell surface proteomics have been changing in the past two decades, from simply global analysis of cell surface proteome for one cell line shift to quantitative proteomics analysis of multiplex samples for biomarker discovery or time- and/or stimulus-dependent cell surface proteomes [99,167,170,173,193,245, 246]. Thus, protocols for the cell surface enrichment should be more high-throughput and automatic to adapt for the multiple sample handing, such as clinical tissues. Besides, quantitative proteomics, including SILAC [27], TMT, isobaric tags for relative and absolute quantitation, LFQ, multiple reaction monitoring /parallel reaction monitoring, data-independent acquisition, plays an important role to characterize the differential cell surface proteomes. Considering the dynamic compositions of the cell surface proteome to adapt the external environment, labeling is better to be performed under more physiological conditions and in vivo labeling is preferential to target the true cell surface proteome. At present, only CCS and cell surface biotinylation have been proven to be useful for in vivo cell surface proteome profiling. Thus, more in vivo labeling strategies need to be developed in the future. Additionally, considering that less than 2% of all determined structures are TMPs [135,247], high-throughput methods should be developed for the prediction of the dynamic stimuli-dependent cell surface protein topology changes. In the future, by integrating fractionated strategies, in-depth cell surface proteome and multiple proteomics analysis, including cell surface proteomics, whole proteomics, phosphoproteomics, genomics, have the potential to be often employed to help gain a more comprehensive understanding of cell surface proteome.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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