Advances in the proteomic profiling of the matrisome and adhesome

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ABSTRACT

Introduction: The matrisome and adhesome comprise proteins that are found within or are associated with the extracellular matrix (ECM) and adhesion complexes, respectively. Interactions between cells and their microenvironment are mediated by key matrisome and adhesome proteins, which direct fundamental processes, including growth and development. Due to their underlying complexity, it has historically been challenging to undertake mass spectrometry (MS)-based profiling of these proteins. New developments in sample preparative workflows, informatics databases, and MS techniques have enabled in-depth proteomic characterization of the matrisome and adhesome, resulting in a comprehensive understanding of the interactomes, and cellular signaling that occur at the cell-ECM interface.

Area covered: This review summarizes recent advances in proteomic characterization of the matrisome and adhesome. It focuses on the importance of curated databases and discusses key strengths and limitations of different workflows.

Expert opinion: MS-based proteomics has shown promise in characterizing the matrisome and topology of adhesome networks in health and disease. Moving forward, it will be important to incorporate integrative analysis to define the bidirectional signaling between the matrisome and adhesome, and adopt new methods for post-translational modification and in vivo analyses to better dissect the critical roles that these proteins play in human pathophysiology.

1. Introduction

In all tissues and organs, cells are surrounded by the extracellular matrix (ECM), which is a highly complex and heterogeneous network of proteins, glycoproteins, and proteoglycans. Each tissue type has a unique ECM composition that provides important structural support, mechanical integrity, and elasticity [1,2]. But the role of ECM goes beyond a simple scaffolding function. By serving as ligands to cell surface receptors, ECM components also provide biophysical and biochemical stimuli influencing fundamental cellular processes, including cell proliferation, migration, or differentiation [3,4]. In addition, various growth factors are sequestered in the ECM, such as epidermal growth factor (EGF), fibroblast growth factor (FGF), or transforming growth factor-β (TGFβ), which can be released by remodeling of the ECM further impacting cellular behavior [1,5]. The capacity of cells to recognize and react to changes in the surrounding ECM is crucial for tissue homeostasis and it is therefore unsurprising that dysregulation of the ECM is associated with various diseases including fibrosis, cancer progression, osteoarthritis, and genetic disorders such as Marfan syndrome [1,6,7].

Cell surface receptors are responsible for transmitting extracellular biochemical and biomechanical cues from the ECM into the intracellular machinery within cells [8]. There is a broad range of different cell surface adhesion receptors, e.g. integrins, discoidin domain receptors, Glycoprotein VI, Endo180, and G-protein coupled receptors, that bind to different ECM ligands [9–14]. The majority of these adhesion receptors (with the notable exception of the discoidin domain receptors) lack intrinsic enzymatic activity for direct signal transmission. Instead, the binding of an ECM ligand to the extracellular domain triggers the recruitment of various interacting proteins to the cytoplasmic tail of the receptor resulting in the formation of large protein complexes known as focal adhesions. The initial complexes are called nascent adhesions (NAs) and connect these receptors to the cytoskeleton [15]. Further maturation of the NAs through recruitment of additional scaffolding proteins, kinases and Rho GTPases form larger focal complexes or very large and more stable focal adhesion complexes [16–18]. By far the most well-studied adhesion receptors are the integrins which are heterodimers of α and β subunits that can interact with a variety of ECM components such as collagens, laminins, or fibronectin [9,19]. Integrins facilitate bidirectional signaling either from ECM into the cell, which is termed as "outside-in" signaling, or in the opposite direction from the cell to the ECM called "inside-out" signaling. "Outside-in" signaling affects a number of cellular processes, including cell shape, migration or differentiation, and is mediated by the recruitment of various kinases and GTPases to form integrin adhesion complexes (IACs) [17,20]. "Inside-out" signaling regulates the affinity of integrins to ECM ligands and is dependent on the interaction of talin and kindlin with the cytoplasmic tail of integrins. These interactions lead to the activation of integrins through conformational changes in the extracellular domain, which enable binding to the ECM ligands [21,22].

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Mass spectrometry (MS)-based methods offer a sensitive and unbiased platform for the analysis of thousands of proteins and are now established as a central analytical tool in proteomics. However, the use of MS-based proteomics for the characterization of the ECM and IAC has been historically challenging due to a lack of consensus on the composition of their protein constituents as well as the limited analytical accessibility of these proteins. To address some of these challenges, recent efforts to integrate genomic and proteomic datasets with extensive literature searches have led to the generation of databases for the "matrisome" [23,24] and "adhesive," [25] which are in silico protein catalogs that comprise ECM and ECM-associated proteins, and integrin adhesion complexes, respectively. In combination with new protocols for the purification and enrichment of matrisome and adhesive components, these developments have led to new insights into the biology of ECM-cellular interactions in multiple disease types over the past decade. In this review, we will describe recent methodological innovations and the latest applications of MS-based proteomics in the comprehensive profiling of matrisome and adhesive proteins. We will also discuss efforts in developing integrative analysis approaches combining both the matrisome and adhesive in order to better understand the complex bidirectional communication between the ECM and cellular adhesion receptors. For readers interested in the biological aspects of the ECM and the functional roles of adhesion molecules, we refer them to these excellent reviews [2,16,26–31].

2. Proteomic characterization of the matrisome

The composition of the ECM has historically been ill-defined. In an effort to better annotate the ECM, Naba et al. sought to create a consensus catalog of proteins that are either part of or are associated with the ECM [23,32]. The authors undertook an analysis of all proteins encoded by genes with ECM specific domains and identified a list of ~1,000 proteins which they termed the "matrisome." These proteins were divided into two classes: the "core matrisome" which comprise glycoproteins, collagens, and proteoglycans; and "matrisome-associated proteins" encompassing affiliated proteins, regulators, and secreted factors (Figure 1) [23]. It should be noted that this is a broad in silico definition that was designed to be inclusive and therefore may include proteins that are predicted to interact with the ECM but whose interactions may not have been experimentally verified or are context-dependent. The first iteration of the matrisome database was constructed for humans and mouse, however in recent years, new databases for other model organisms including Drosophila melanogaster [33], Danio rerio [34] and Caenorhabditis elegans [35] have been added. In parallel, the MatrixDB bioinformatic tool has been developed for the analysis of interaction networks among ECM proteins, proteoglycans, and polysaccharides [36,37]. MatrixDB combines experimental data with interactions reported in multiple databases and thus allows in silico investigations of ECM organization as well as reported interactions between ECM components and adhesion molecules.

Proteomic analysis of the matrisome by MS is challenging due to the inherent physico-chemical properties of these proteins, which are incompatible with conventional sample preparation protocols. MS-based proteomics rely on the enzymatic digestion of proteins solubilized in aqueous solvents. However, matrisomal proteins and particularly core matrisomal proteins have large molecular weight and are extensively cross-linked, which collectively result in low solubility [38]. In contrast to most intracellular proteins, the core matrisomal proteins remain insoluble even in high concentrations of salts and detergents. As a result, one key limitation of conventional sample preparation workflows is that the majority of ECM proteins are removed with the cell debris. Reliable detection and quantification is made more challenging by the wide dynamic range of matrisomal proteins spanning over >5 orders of magnitude from highly abundant collagens to low abundance secreted and regulatory factors [39,40]. Taken together, these limitations hinder the analytical accessibility of matrisomal proteins for MS-based profiling. Various biochemical methods have since been developed to overcome these limitations [41].

In general, two major workflows have been established: 1) ECM enrichment-dependent and 2) Enrichment-independent methods (Figure 2). ECM enrichment-dependent approaches can be further divided into decellularization methods and sequential extraction methods (Figure 2). In this section, we will describe each of these methods, summarize their advantages and disadvantages, and compare their performance in enabling matrisome characterization.

![Figure 1](image)

**Figure 1.** a) Composition of the human in silico matrisome. b) Selected representative proteins for each matrisomal class.
2.1. Enrichment-dependent approaches – decellularization

Tissue decellularization was originally developed for the purposes of bioengineering and regenerative medicine [42]. This technique exploits the insoluble nature of core matrisome proteins and is based on the disruption and removal of cells from intact tissue specimens, which leaves behind an insoluble ECM scaffold for downstream analysis (Figure 2). The removal of the cells from intact tissue can be achieved by physical, chemical, or biochemical methods [43]. Physical methods include freeze-thaw cycles, agitation, pressurization or sonication [44]. Chemical treatment often uses a variety of agents to disrupt the cell wall. For instance, detergents such as sodium dodecyl sulfate (SDS) [45], Triton X-100 [46] or 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) [47] have been successfully applied for decellularization of various tissue types. Alternatively, cell wall disruption can also be achieved by the application of osmotic pressure using hypertonic or hypertonic solutions [48,49]. Treatment by acids or bases can be also used for the perforation of biological membranes, however these acid-base methods are not suitable for proteomic applications due to the disintegration of the ECM resulting in the loss of matrisomal proteins [49].

Biochemical methods employ specific enzymes for enrichment of ECM protein components by reducing contaminants from other biomolecules. Examples include benzonase or DNase for the degradation of nucleic acids and PNgase F for the removal of the N-linked oligosaccharides [43,50]. Physical, chemical, and biophysical methods are often combined to maximize enrichment efficiency. For instance, a typical decellularization protocol can start with freeze-thawing the sample followed by several washing steps in hypertonic buffer containing detergents while constantly agitated. A decellularization procedure can then be completed by incubation of the resulting ECM scaffold in benzonase. These scaffolds can then be solubilized and homogenized in buffers with chaotropic agents such as urea, thiourea, or guanidine hydrochloride prior to proteolytic digestion and MS analysis.

Decellularization techniques have been demonstrated to be highly effective, particularly for the enrichment of core matrisomal proteins [41,51–53]. For instance, an analysis of matrisomal proteins in decellularized murine lungs by Calle et al. showed almost complete preservation of collagens and laminins when compared to native specimens [51]. In contrast, the vast majority of soluble matrisome-associated proteins are typically lost during the decellularization process, which makes this method largely unsuitable for deep analysis of this matrisome compartment [41,51]. In another example, Leng et al. analyzed matrisomal proteins in porcine skin samples decellularized by combining biochemical and chemical methods [54]. Quantitative analysis showed strong enrichment of collagens and glycoproteins in decellularized samples compared to the native tissue, however there was a reduction in the majority of matrisome-associated proteins and several proteoglycans after enrichment. The composition of washing buffers, particularly the type of detergent used has a strong effect on the types of proteins enriched [41,42,55,56]. Less aggressive detergents such as Triton X-100 or sodium deoxycholate (SDC) allow milder decellularization resulting in better preservation of matrisomal proteins and a higher number of identified matrisomal proteins compared to more aggressive detergents such as SDS or CHAPS. On the other hand, stronger detergents decrease contamination by intracellular proteins and thus provide higher purity of matrisome components in subsequent MS analyses [41,51]. More recently, Mayorca-Guiliani et al. developed a novel method for in-situ...
decellularization of tissue termed iSDoT, which uses the native vascularature of the tissue for delivery of the decellularizing agents consisting of SDC and Triton X-100 based buffers [57]. The authors demonstrated that iSDoT efficiently removed cellular components but kept the original architecture of collagen fibrils preserved in murine mammary fat pad, lungs, liver, and lymph nodes. In contrast, ex vivo SDS decellularization resulted in disruption of the structure and organization of collagen. Therefore, the iSDoT method can be beneficial for multimodal studies of ECM proteins comprising of proteomic profiling of matrisome components and interrogation of their spatial distribution by imaging analysis.

2.2. Enrichment-dependent approaches – sequential extraction

In contrast to decellularization, sequential extraction methods apply the ECM enrichment steps after homogenization of the tissue sample (Figure 2). Soluble proteins are then removed from the homogenate by several washing steps in a sequential manner. Washing buffers with different compositions are used to maximize enrichment efficiency and improve the purity of the resulting insoluble fraction containing ECM proteins. The first application of this method by Naba et al. used a commercial Compartmental Protein Extraction Kit to identify over 100 matrisomal proteins in fresh frozen murine lung and colon tissue samples [23]. This kit employs different buffers for solubilization and extraction of cytosolic, nuclear, membrane, and cytoskeletal proteins while matrisomal proteins remain in the insoluble pellet, which is subsequently enzymatically deglycosylated, and digested for MS analysis. This method has since been successfully applied to a number of other tissue types including mouse brain [58], liver [58], mammary gland [58,59], human pancreas [60] and omentum biopsies from patients with high-grade serous ovarian cancer (HGSOC) [61].

More recently, a number of alternative protocols based on similar principles have been developed [53,62–65]. For instance, the Hansen laboratory has published a method based on the removal of cellular proteins using a “high salt buffer” containing 3 M NaCl and 0.25% CHAPS [53]. ECM proteins were then extracted from the resulting insoluble pellet by a chaotropic agent (8 M urea), leading to the generation of a chaotrope-soluble ECM fraction (sECM). The authors further introduced a chemical digestion step for the remaining insoluble pellet by using cyanogen bromide (CNBr) to improve the extraction yield of the chaotrope-insoluble ECM proteins (iECM). With this approach, quantitative proteomic analysis confirmed a strong enrichment of the matrisomal proteins in both the sECM and iECM fractions compared to the cellular fraction extracted by the high salt buffer. The protocol has since been used for the analysis of matrisome from rat lung [53], human skin [66], breast cancer biopsies [67] and mouse fibroblastic reticular cells [68]. In the next iteration of the protocol, the authors used hydroxylamine instead of CNBr to chemically digest the iECM fraction [69]. A comparative study performed across five different mouse tissue types showed no increase in the number of detected matrisomal proteins using the hydroxylamine method compared to CNBr, but the hydroxylamine treatment increased the peptide signals of core matrisomal proteins in the iECM fraction as measured by selective reaction monitoring MS. Moreover, digestion by hydroxylamine is safer than treatment with CNBr. Alternatively, enzymatic digestion can be used instead of chemical digestion. For instance, the use of the Liberase enzymatic blend of collagenases has been introduced as an additional step prior to cleavage by trypsin in an extraction protocol developed by Ouni et al. [64]. Here, cellular proteins were first extracted by 0.3 M NaCl and an MS-compatible detergent with the remaining insoluble pellet digested by Liberase prior to MS analysis.

Other modifications to the sequential enrichment approach include a study by Schiller et al., who developed an extraction method termed quantitative detergent solubility profiling (QDSP) which uses a sequential set of buffers with increasing concentration of both detergents and NaCl [62]. In this protocol, the sample is separated into three soluble fractions and an insoluble pellet which is further mechanically disintegrated and ultrasonicated in the presence of proteolytic enzymes LysC and trypsin to digest the matrisomal proteins. QDSP was employed in the proteomic study of mouse lung tissue repair after treatment with bleomycin. The authors performed a temporal comparison of the tissue repair and identified potential time-dependent shift in the solubility of several matrisomal proteins [62]. In another study, Knott et al. developed a fast ECM-enrichment method using a novel detergent 4-ethylphenylazosulfonate (Azo) which can be rapidly degraded by ultraviolet irradiation and is therefore compatible with MS [70,71]. In this method, cryo-pulverized tissue samples of mouse mammary tumors were treated with Triton X-100 for 45 min, and the soluble protein fraction was acetone precipitated to remove Triton X-100 and subsequently resuspended in buffer with the Azo detergent. The Triton X-100 insoluble pellet containing the core matrisomal proteins was homogenized in Azo buffer to completely solubilize the pellet. Both soluble and insoluble fractions were further processed by conventional in-solution digestion and irradiated by ultraviolet light to degrade the Azo detergent prior to MS analysis. Using this approach, the authors demonstrated that the use of Azo buffer for solubilization of the insoluble fraction significantly increases the relative protein abundance of collagens compared to extraction by either 8 M urea or 25 mM ammonium bicarbonate.

The Mayr laboratory has developed a 3-step extraction protocol for enrichment of matrisomal proteins [72]. In the first step, samples are extracted by 0.5 M NaCl followed by extraction using 0.08% SDS in the second step. In the last step, samples are solubilized in 4 M guanidine hydrochloride and further processed by a conventional proteomic workflow. This method was successfully applied to the matrisome characterization of human aorta [72], human atherosclerotic plaques [73] and for the analysis of ischemia-related matrisome remodeling in porcine myocardium [74]. To further extend its capabilities to the glycoproteomic analysis of the matrisome, the 3-step extraction protocol has been successfully combined with lectin-based affinity chromatography for enrichment of glycoproteins [50,75]. The authors employed glycoprotein enrichment kits with 1:1 mixture of Concanavalin A and Wheat Germ Agglutinin to enrich for matrisomal glycoproteins.
from the samples after the 3-step extraction. Subsequent analysis of the enriched glycoproteins by MS successfully identified 65 glycosites on 35 matrisomal proteins from the human left and right atria [75].

2.3. Enrichment-independent methods

As discussed above, decellularization and sequential extraction are effective in the enrichment of core matrisomal proteins, however, the more soluble matrisome-associated proteins are often lost during the enrichment process resulting in the lack of detection or underestimation of the levels of this class of proteins. To preserve the soluble components of matrisome and minimize the number of sample processing steps required, enrichment-independent methods that directly analyze the matrisome by MS-based proteomics have been developed [40,76–78]. These methods use homogenized samples but in contrast to conventional lysis protocols, the cell debris and insoluble fraction are not removed from the sample to minimize any losses of matrisomal proteins (Figure 2).

Surfactant and Chaotropic Agent-assisted Sequential Extraction/On-Pellet Digestion (SCAD) is a direct method for sample processing developed by the Li laboratory [77]. In the SCAD protocol, cells are first lysed by sonication in the presence of a high concentration of SDS (4%) and subsequently heated to 95°C. In the next step, all proteins in the lysate are reduced and alkylated before overnight precipitation by acetone to remove the MS-incompatible SDS. The precipitated pellet is washed and dried prior to addition of a chaotropic buffer containing urea and trypsin digestion. The authors tested the SCAD protocol on the human breast cancer cell line MDA-MB-231 and identified 32 core matrisomal and 89 matrisome-associated proteins by single-shot analysis. Comparison of SCAD with two conventional sample processing methods showed no differences in numbers of identified matrisomal proteins, however intensity-based absolute quantification (iBAQ) analysis revealed ~10% increase in quantity of matrisomal proteins in the sample prepared by SCAD. The Huang laboratory has previously employed direct methods for the analysis of matrisomal proteins in mouse heart, liver, lung and mammary glands [41,76]. Tissues were homogenized and lysed in 8 M urea buffer [41] or in 3 M NaCl buffer with CHAPS [76]. After acetone precipitation, pellets were resuspended in 8 M urea, reduced, alkylated and digested by trypsin in solution or by gel-assisted digestion protocol to remove residual CHAPS. They showed that enrichment-independent methods using the 8 M urea homogenization buffer outperformed Triton X-100- and SDS-based decellularization, Compartmental Protein Extraction kit and “high salt buffer” enrichment methods in the number of identified matrisome-associated proteins [41].

A detergent-free sample processing method termed Sample Preparation by Easy Extraction and Digestion (SPEED) has been recently developed by Doellinger et al. [78]. SPEED relies on the acidification of samples by concentrated trifluoroacetic acid, which effectively lyases and solubilizes both cellular and tissue samples. The authors tested SPEED on a range of samples, including cultured cells and lung tissue, and demonstrated the full dissolution of samples. In the next step, samples are brought to neutral pH and further processed by a conventional proteomic workflow consisting of reduction, alkylation and digestion of proteins by trypsin. When compared with detergent-based filter-assisted sample preparation (FASP) [79], single-pot, solid-phase-enhanced sample preparation (SP3) [80] and a chaotropic agent-based (8 M urea) protocol, SPEED more than doubled protein yields obtained from mouse lung samples and improved quantitative reproducibility across all samples, which may be attributed to the lower number of sample handling steps. The SPEED protocol also increased the overall number of proteins identified in the samples, however, analysis of the matrisome subset found that the number of identified matrisomal proteins did not differ between individual methods.

Fibrosis is a pathological condition where normal tissue is replaced by ECM [81]. In the normal wound healing process, ECM is temporarily deposited to create a scar and later degraded when the original tissue is restored. However, in the case of repeated injuries, e.g. chronic inflammation, tissue repair is dysregulated resulting in excessive accumulation of ECM, disruption of the tissue architecture and loss of function, which may lead to organ failure. Tian et al. used FASP to study the matrisomal profile of lungs with idiopathic pulmonary fibrosis (IPF) [82]. FASP is a widely used sample processing proteomic method for detergent removal and protein digestion based on the retention of intact proteins while smaller peptides can permeate through the filter membrane [79]. The authors analyzed normal human lung versus IPF and successfully detected 229 matrisomal proteins including 104 core matrisomal proteins and 125 matrisome-associated proteins. Quantitative comparison revealed 56 matrisomal proteins with differences in expression levels between IPF and normal samples including galectin 7 (LGALS7), tenascin C (TNC), cathepsin B (CTSB) as well as number of collagens and laminins. This shows that levels of matrisomal proteins may be used for the detection and monitoring of fibrosis. For instance, increased blood levels of pro-peptides for collagen III (PRO-C3) and collagen VI (PRO-C6) have been detected in patients with IPF when compared to healthy controls and PRO-C3 and PRO-C6 levels are also correlated with the IPF progression [83]. In another study, PRO-C3 serum levels were identified as a promising diagnostic and prognostic biomarker of liver fibrosis [84,85].

2.4. Comparison of matrisome proteomic workflows

As described above, a number of ECM enrichment-dependent and – independent protocols have been developed and several comparative studies have been performed. Krasny et al. have evaluated two decellularization methods and two sequential extraction protocols across four types of murine tissue in terms of enriched protein yield, number of detected matrisomal proteins, and purity of the enriched matrisome [41]. In general, all four methods successfully preserved the core matrisomal proteins in all tissue samples tested with the highest number of proteins identified in liver tissue. The authors further compared these enrichment-dependent strategies with an enrichment-independent method and showed that non-enriched samples consistently contained more soluble matrisome-associated
proteins than either decellularized or sequentially enriched samples. This loss of matrisome-associated proteins during enrichment was later confirmed in a follow-up quantitative analysis of enriched and non-enriched mouse liver and lung tissue samples using sequential window acquisition of all theoretical spectra (SWATH)-MS [76]. Comparative assessment of the enrichment methods revealed that SDS decellularization provided the highest purity of matrisome enrichment with the lowest level of intracellular protein contamination. On the other hand, the highest numbers of matrisomal proteins were detected in samples enriched by the “high salt buffer” extraction protocol developed by the Hansen laboratory described earlier.

More recently, McCabe et al. performed a detailed assessment of several methods for enrichment of the core matrisome from whole mouse powder [86]. For the purposes of evaluation, the authors divided the sample processing workflow into two parts: 1) removal of the soluble proteins and 2) the solubilization and digestion of the enriched insoluble fraction; and assessed these two parts independently. In the first step, five protocols for soluble protein removal were compared including the Triton X-100 decellularization, Compartmental Protein Extraction Kit, “high salt buffer”protocol, QDSP and a 2-step method combining buffers from QDSP and “high salt buffer” protocol. No significant differences between the methods were found in the enrichment of collagens while Triton X-100 decellularization and the QDSP method detected slightly lower number of glycoprotein and proteoglycan peptides. Next, four methods of solubilization and digestion were evaluated using either whole mouse powder or four murine organs enriched by the “high salt buffer” protocol. A combination of solubilization in 6 M guanidine hydrochloride with hydroxylamine digestion outperformed the other three methods in terms of the number of identified core matrisomal proteins, unique peptides and peptide-to-spectrum matches. The addition of PNGase F during the optimization of the solubilization/digestion step further improved coverage of glycoprotein and proteoglycans.

In summary, enrichment-independent methods offer fast and straightforward sample processing for subsequent proteomic analysis of the global proteome including the matrisome, with minimal losses of core matrisomal or matrisome-associated proteins (Table 1). On the other hand, the high complexity of the sample obtained by enrichment-independent methods may require the introduction of additional off-line fractionation steps to improve proteomic depth (Table 1). Enrichment-dependent methods have been shown to be very effective in preserving core matrisome proteins and therefore a single-shot analysis may provide better coverage of this matrisome class compared to non-enriched samples, particularly in tissues with very low ECM content such as liver. Overall, the selection of the optimal method for analysis of matrisome proteins is dependent on the experimental question, tissue type of interest, and the class of matrisomal proteins under study.

2.5. Mass spectrometry developments in the analysis of the matrisome

In addition to developments in databases and sample preparative workflows, there have also been developments in MS methodologies for the analysis of the matrisome. These include technologies to increase the reproducibility of matrisomal proteins identified between MS experiments. Conventional data-dependent acquisition (DDA) MS is plagued by low reproducibility between experiments due to the stochastic nature of precursor peak selection and fragmentation [87]. Targeted methods such as single reaction monitoring MS (SRM-MS) or data-independent acquisition MS (DIA-MS) are alternative MS data acquisition methods that offer very high reproducibility in the identification of proteins between experiments and better sensitivity [88,89]. A targeted method for absolute quantification of matrisomal proteins has previously been reported by the Hansen laboratory [53]. In this method, quantification concatamer (QconCAT) – a 13C labeled artificial protein constructed from 76 concatenated peptides representing 54 matrisomal proteins is added to the ECM enriched sample prior tryptic digestion. The digested sample is then quantified by SRM-MS using the peptides from the artificial protein as an internal heavy labeled standard. This approach has been successfully applied to the analysis of the matrisome in rat lungs [53], murine liver and mammary glands [39] and human myocardial ECM [90].

Compared to targeted methods such as SRM-MS, DIA-MS offers increased proteomic depth (hundreds to thousands of proteins) while maintaining exceptional reproducibility. To date, several studies that have employed DIA-MS for matrisome analysis have been published. For instance Rolandsson Enes et al. performed quantitative characterization of matrisome produced by bone marrow- and lung-derived mesenchymal stem cells (MSCs) [91]. Using DIA-MS analysis of MSCs and conditioned media, the authors quantified 234 matrisomal proteins in total and revealed characteristic matrisomal profiles for bone marrow- and lung-derived MSCs. Krasny et al. generated a DIA spectral library containing 85 core matrisomal and 116 matrisome-associated proteins that has been used in the analysis of mouse liver and lung matrisome [76].

Matrix-assisted laser desorption/ionization (MALDI) imaging is an interesting application that can provide two-dimensional (2D) distribution of matrisomal proteins in tissue [92,93]. This method is based on application of proteolytic enzymes such as trypsin, elastase or collagenases on histological tissue sections with subsequent analysis of the released peptides by MS. In a method developed by Angel et al. collagenase III, elastase or matrix metalloprotease 12 were sprayed on a wide range of tissue samples including liver, intestine, aortic valve and breast needle core biopsies to digest matrisomal proteins [92,93]. The resulting peptides were analyzed by MALDI imaging which led to the generation of 2D maps of various collagens, fibronectin or elastin with a spatial resolution of 25 µm. Distribution maps with this level of detail can be complemented with optical imaging analysis such as laser scanning confocal microscopy (LSCM), spinning disc confocal microscopy (SDCM) or super-resolution method of stimulated emission depletion (STED) that can provide more precise information about the structure of the ECM and cellular component within the sample [94]. The major disadvantages of MALDI imaging are low proteome coverage and unreliable protein identification, which stems from the low efficiency of gas-phase fragmentation [95]. To address this challenge, a method that can reliably detect hundreds of proteins from the tissue has been developed by
Raghunatan et al. [96] The authors applied small droplets with enzymes on specific locations of the tissue to digest proteins and extract released peptides [96]. Moreover, this method can be easily extended to glycomic or glycoproteomic analysis by the simple addition of another digestion step with specific enzymes such as chondroitinase, heparinases or PNGase F that allow identification of glycosyls in proteoglycans. On the other hand, the size of the droplets significantly limits spatial resolution that can be achieved and increases the overall time required for the procedure. Droplets with released analytes are subsequently processed and analyzed by LC-MS /MS. Using this approach, the authors performed proteomic and glycomic analysis of brain tissue samples and identified the upregulation of various collagens, proteoglycans and annexins in brain samples with Parkinson’s disease when compared normal tissue [97].

### 3. Proteomic characterization of adhesome

Similar to the matrisome, an insufficient clarity of the composition of the adhesome has hindered the study of the role and interactions of individual components within adhesion complexes. To address this drawback, the Geiger laboratory has performed a deep literature mining exercise and compiled a “literature-curated adhesome” consisting of 151 proteins involved in integrin-mediated adhesions [25]. Subsequent bioinformatic analysis of protein-protein interactions allowed the construction of the first adhesome network showing mutual relationships between individual components of the adhesome. This initial list of proteins has been updated as new discoveries have been made and the most recent version of the adhesome network contains 232 proteins with over 6,500 protein-protein interactions (Figure 3) [98]. To indicate the functional role of the proteins within the IAC, individual components of the adhesome network are classified into intrinsic proteins or associated proteins based on their localization. Intrinsic proteins are the core IACs proteins while associated proteins interact with the intrinsic proteins and regulate their activity. This classification is, however reliant on available localization data, which might be incomplete. Intrinsic and associated proteins are further classified based on their function within the complex into several categories such as adaptors, adhesion receptors, kinases, regulators, etc. Many aspects of this in silico constructed adhesome network requires experimental confirmation of both the identity and the mutual interactions of proteins included in the database, which is an active area of research being pursued by several research groups. MS-based proteomics has become an important tool for validating the in silico adhesome network. This is reflected by a comprehensive study where the combination of seven published proteomic datasets resulted in defining a “meta-adhesome” consisting of >2,400 proteins which have been identified in IACs by MS [8,99]. This “meta-adhesome” likely contains protein contaminants that do not participate in the actual ECM-mediated adhesions and was therefore further refined into a core of 60 components identified in at least 5 of the 7 datasets, which were termed as the “consensus integrin adhesome.”

The low stability of adhesion complexes and the transient dynamic changes of protein components within complexes, as
well as the hydrophobic nature of the transmembrane adhesion receptors are major analytical challenges in the proteomic characterization of adhesome [100,101]. For instance, imaging experiments have revealed that the assembly and disassembly of adhesion complexes is a highly dynamic process mediated by weak protein-protein interactions [101,102]. Moreover, the low solubility of the transmembrane domains of adhesion receptors limits the ability to isolate adhesion complexes by conventional proteomic sample preparative protocols which would either result in loss of the insoluble fraction or loss of protein-protein interactions within adhesion complexes. Three major workflows has been developed and successfully applied in the proteomic analysis of the adhesome (Figure 4): 1) Protein cross-linking, 2) hypotonic shock and 3) Proximity-dependent biotinylation (BioID), which will be discussed in detail in the next section.

3.1. Protein cross-linking

Protein cross-linking utilizes membrane-permeable chemical reagents that specifically modify and cross-links individual components of the assembled adhesion complex, capturing and preserving the complex during cell lysis and protein extraction (Figure 4, Table 2) [103–107]. Current cleavable cross-linkers that are used include dimethyl-3,3′-dithiobispropionimidate (DTBP) which do not interfere with conventional proteomic workflows. On the other hand, the low specificity of the cross-linking reaction leads to a high level of nonspecific contaminants, which requires the judicious use of appropriate negative controls to distinguish true interactors from contaminants (Table 2). To further preserve the integrity of adhesion complexes, rapid but mild procedures are applied for the isolation of adhesion complexes [101]. For instance, weak detergents combined with
sonication or buffers with low ionic strength have been employed for gentle cell lysis \[104,108–110\]. In an early example of this approach developed by Humphries et al., the authors used paramagnetic beads coated with the integrin ligands to induce the formation of adhesion protein complexes in a suspension of cells \[108\]. Formed complexes were then stabilized by the DTBP cross-linker followed by cell lysis using sonication in a Triton X-100 lysis buffer prior to MS-based proteomic analysis. This led to the discovery that the regulator of chromosome condensation 2 (RCC2) protein was a key component of fibronectin (FN)-induced adhesion complexes with a key regulatory role in cell migration.

In an extension to the protocol to facilitate the study of phosphorylation-mediated signaling within integrin adhesome complexes, the same group added a phosphopeptide enrichment step after adhesion enrichment prior to MS-proteomics analysis \[106\]. The authors identified 50 phosphoproteins in FN-induced adhesion complexes including 10 phosphoproteins whose non-phosphorylated forms were not previously detected by global proteomic studies despite being defined as components of the literature-curated adhesome network. Finally, this methodology was further used to distinguish between adhesion complexes that are associated with active and inactivate integrin subunits \[107\]. Here, the authors used paramagnetic beads coated with activation-state specific anti-integrin antibodies to specifically enrich for proteins participating in activated integrin complexes. Quantitative proteomic analysis of the enriched samples identified activation state-specific recruitment of proteins into the adhesion complexes with a number of adaptor proteins (e.g. talin, ezrin, vinculin, Crk-like protein) and actin regulators (e.g. actin-related protein 2/3, filamins, microtubule-actin cross-linking factor 1) upregulated in active integrin complexes. Surprisingly, several proteins involved in mitosis, cell division or RNA splicing and transport that are not classically known to be associated with adhesion signaling were found to be upregulated in the active integrin complexes indicating previously undescribed signaling complexity within the adhesion sites and their involvement in various downstream biological processes.

### Table 2. Relative merits of proteomic approaches for analysis of the adhesome.

| Method       | Advantage                           | Disadvantage                                    |
|--------------|-------------------------------------|-------------------------------------------------|
| Cross-linking| Simple                              | High level of nonspecific contaminants          |
|              | Cleavable cross-linkers compatible with MS | Crucial selection of appropriate negative control |
|              | Stabilization of the adhesion complex|                                                 |
| Hypotonic shock| Simple                             | Ion suppression effects by co-enrichment of highly abundant proteins (fibronectin, actin) |
|              | Can be combined with cross-linking or BioID | High level of intracellular contaminants |
|              | Compatible with western blot or imaging analysis | Low reproducibility |
| BioID        | Can be targeted to specific components of the adhesion complexes | Requires stable expression of bait-BirA* protein in cells |
|              | Inducible system                    | Potential steric hindrance                       |
|              | Compatible with imaging analysis    | Not suitable for time-sensitive experiments      |
|              |                                     | High level of nonspecific contaminants due to BirA* promiscuity |

#### 3.2. Hypotonic shock

Kuo et al. developed a rapid method based on hypotonic shock to isolate adhesion complexes from fibroblasts grown on FN coated plates (Figure 4) \[109,110\]. In this method, fibroblasts are covered with hypotonic buffer for 3 min which is sufficient to initiate cell swelling. The swollen cells and cytoplasmic membranes are then washed away by hydrodynamic forces while adhesion complexes remain attached to the fibronectin coating and can be collected for further processing by conventional proteomic protocols. One disadvantage of this approach is that reproducibility appears to be relatively low with only 38% of proteins reliably identified in two out of three repeats. In addition, removal of highly abundant actin and fibronectin by immunoprecipitation is recommended to prevent suppression effects during MS data acquisition and to improve the detection of low abundant adhesome components \[110\] (Table 2). Using this approach, Kuo et al. identified 283 expected adhesosomal proteins and 471 potential new focal adhesion components in human foreskin fibroblasts \[109\]. More recently, the same isolation technique has been combined with cross-linking and applied to study focal adhesions in glomerular epithelial cells (podocytes) where quantitative proteomic analysis of the enriched and cross-linked adhesion sites identified Band 4.1-like protein 5 (EPB41L5) as a potential key component of IACs \[105\]. Subsequent experiments revealed a crucial role for EPB41L5 in the maturation of IACs and actomyosin contractility through interaction with Rho guanine nucleotide exchange factor 18 (ARHGFE18).

The ECM ligand-specific differences in adhesion site assembly and resulting changes in signaling and cell morphology were investigated in a recently published study by Randles et al. \[111\]. The authors observed significant differences in the cell shape of podocytes grown on three different ECM proteins, collagen IV, laminin 511 and laminin 521 that serve as ligands for integrins. Immunofluorescence and western blot analysis also found differences in cell signaling pathways where Proto-oncogene tyrosine-protein kinase Src (Src), FAK and Ras-related C3 botulinum toxin substrate 1 (Rac1) activation was enhanced on collagen IV when compared to the laminins. Proteomic analysis of the adhesion complexes enriched from cells revealed distinct proteomic profiles for collagen IV and laminin ligands. For instance, different adhesome components suppressing Rac1 activity were detected in adhesion complexes when cells were exposed to distinct ECM ligands suggesting ligand-specific regulation of this enzyme. In addition, protein kinase C alpha (PKCα) and integrin α3 were significantly upregulated in laminin-mediated but not collagen-mediated complexes. Subsequent experiments confirmed that the interaction between laminins and integrin α3 results in the activation of PKCα, which is responsible for the observed elongated cell shape of podocytes.

#### 3.3. Proximity-dependent biotinylation (BioID)

BioID is an elegant method for labeling and enrichment of interacting proteins and thus an attractive tool for proteomic analysis of adhesion complexes (Figure 4) \[112–114\]. BioID
uses a promiscuous mutant of the biotin ligase BirA* fused with the target of interest – a bait. In the presence of biotin, proteins close to the bait are biotinylated by BirA* and the resulting biotinylated proteins are subjected to affinity purification and MS-based proteomics. There are some limitations to the BioID method, for example, unlike the cross-linking approach, there is a requirement to first engineer cells with stable endogenous expression of the bait-BirA* protein (Table 2). BirA* can modify any protein in an accessible distance from the bait which may result in high levels of nonspecific contaminants. Furthermore, the presence of BirA* in the fusion protein can sterically block endogenous protein-protein interactions and time required for the biotinylation reaction makes it challenging for BioID to be used in experiments where interactions occur at short time scales. Nevertheless, BioID has been successfully employed by Dong et al. to investigate the interacting partners of two adhesin bait proteins, paxillin and kindlin-2 [184]. Apart from known adhesion components, the authors also identified seven new proteins that directly interact with paxillin and two novel interactors of kindlin-2 including KN motif and ankyrin repeat domain-containing protein 2 (Kank2) that was found to interact with both bait proteins. Therefore, Kank2 may serve as an adaptor protein bridging paxillin and kindlin-2 in adhesin complexes. In addition, the absence of biotinylation on FAK in the experiments with BirA*-kindlin-2 contradicts several theoretical models of focal adhesion complexes that predicts close proximity between FAK and the cytoplasmic membrane. Kindlin-2 interacts directly with the short cytoplasmic tail of integrins therefore any proteins in close proximity to integrins and the cytoplasmic membrane would have been labeled by BirA*. The lack of FAK biotinylation in this study has led to the construction of new models of focal adhesion complexes with FAK placed further away from the cytoplasmic membrane.

The BioID approach has been greatly expanded in a recent study where 16 commonly identified adhesome components were used as baits to decipher the architecture of IACs by MS-based analysis of proteins interacting with individual baits [114]. By combining data from all 16 proteomic datasets, the authors constructed a network of 147 adhesome proteins with 361 interactions. Bioinformatic analysis of the constructed network revealed five protein-protein interaction clusters with a central cluster encompassing the protein paxillin which serves as a critical link with all the other clusters. By contrast, peripheral clusters shared only a small number of direct interactions. The structure and composition of these five clusters overlap with the recent topological models of IAC obtained by imaging experiments [115–117] and likely serve as functional units with specific roles within the complexes.

All three methods (cross-linking, hypotonic shock, BioID) combined with MS-based proteomics have proven their ability to provide biologically relevant information about the composition of individual adhesion complexes and interactions between individual IAC components. Cross-linking and hypotonic shock coupled with MS profiling are relatively straightforward methods for proteomic analysis of the IAC and has facilitated the construction of “integrin consensus adhesome.” BioID can offer more detailed insight into the organization and topology of the adhesion complexes but requires prior knowledge of the bait as well as the need for engineering BirA*-bait cell lines. Taken together, these methods represent well-established platforms for enrichment of adhesion complexes prior to proteomic analysis.

4. Integrative analysis of the matrisome and adhesome

Given the recent advances in the in silico definition and methodological developments in the proteomic profiling of both the matrisome and adhesome, the field is primed to undertake integrative proteomic analysis combining parallel studies of the matrisome and adhesome on the same sample. Such approaches can help to define the dynamic and bidirectional alterations in matrisome remodeling and adhesome signaling that occur in physiological and disease processes. Such integrative analysis offers a unique opportunity to investigate these reciprocal events with unprecedented detail.

To date, very few integrative analyses have been reported. One recent example describes the role of EPB41L5 in the regulation of integrin adhesion complexes in podocytes and related ECM remodeling [118]. The authors performed quantitative proteomic analysis of three components (IACs, soluble and insoluble matrisome) in normal and EPB41L5-deficient podocytes. For the quantitative analysis of IACs, the authors used cell labeled by stable isotope labeling using amino acids in cell culture (SILAC) and combined cross-linking with the enrichment method developed by Kuo et al. Mutual comparison of IACs from normal and EPB41L5-deficient cells identified EPB41L5-dependent recruitment of PDZ and LIM protein 5 (PDLIM5) and alpha-actinin 4 (ACTN4) into the adhesion complexes. Both PDLIM5 and ACTN4 participate in acto-myosin contractility. In addition, EPB41L5-deficient podocytes exhibited loss of ECM-cell adhesion due to insufficient maturation of integrin adhesion sites. The authors also analyzed the conditioned media and insoluble ECM produced and deposited by the cells on the plate (ECM deposits) to study proteomic changes in the soluble and insoluble matrisome, respectively. Proteins in conditioned media were extracted by precipitation with trichloroacetic acid, while ECM deposits were collected, decellularized by Triton X-100, washed in RIPA buffer and then denatured in Laemmli buffer at 70°C. MS analysis of conditioned media and ECM deposits identified 402 matrisomal proteins and revealed significant changes in the distribution of core matrisome proteins between the condition media and ECM deposits. In particular, the loss of EPB41L5 led to decreased levels of nidogen-2, laminin α5 and laminin γ1 in the insoluble ECM, however levels of these components were increased in the conditioned media. The authors hypothesize that the interaction between integrins and ECM components is needed for the initiation of polymerization and self-assembly of the matrisomal proteins such as collagen IV and laminins into the basement membrane structure. Therefore, the lack of initiation points on the surface of the EPB41L5-deficient cells resulted
in increased levels of usually polymerized insoluble matrismo
mal proteins in the conditioned media.

5. Conclusion

Historically, the use of conventional proteomic approaches for the analysis of ECM proteins and adhesion complexes has been challenging. However, recent technological, methodological, and bioinformatic advances in proteomics has opened new avenues for the profiling of these two challenging pro-
tecmic compartments. In this review, we have summarized developments in the accurate annotation of both the matri-
some and adhesome which will enhance our understanding of their composition and protein-protein interactions. We antici-
pate that moving forward, these methods will provide a com-
prehensive proteomic toolkit for the interrogation of complex interactions between the cellular adhesion machinery with their cognate matrissome environment.

6. Expert opinion

One of the major limitations of the current methodologies for adhesome analysis is the reliance on in vitro experiments which may not recapitulate biologically relevant conditions in vivo. For instance, cells are routinely plated on single sub-
strates (e.g. collagen, fibronectin) which do not resemble the complex ECM network naturally present in the tissue. With recent innovations in the application of BioID in animal models [119,120], we anticipate that there will be future exciting opportunities to utilize this method to study adhesion complexes under physiological conditions. Furthermore, new developments in DIA-MS approaches that harness species-
specific spectral libraries to interrogate matrissome remodeling and adhesion signaling in mouse xenograft models will further push the boundaries of in vivo analysis of the adhesome and matrissome moving forward [121].

With the exception of a few published studies, the field has yet to develop accurate methods for the analysis of post-
translational modifications (PTMs) such as glycosylation and cross-linking in the proteins that comprise the matrissome [122]. These PTMs are key regulators of conformation, functiona-
ility or stability of proteins and therefore a deeper under-
standing of the PTM events within matrissome proteins can shed light on their biological functional and mechanistic reg-
ulation. For instance, genomic analysis of the mutational bur-
den of the 9,075 cancer patients that were included in the Pan-Cancer cohort of The Cancer Genome Atlas consortium revealed PTM-affecting mutations that can potentially alter interactions of matrissomal proteins and thus have negative effects on the structure, stability, and signaling capability of the tumor matrissome [123,124]. Alterations in PTM levels in matrissomal proteins have already been found in pathophysio-
logical processes such fibrosis or various cancer types. For instance, expression of several collagen cross-linking enzymes has been detected upon treatment with transforming growth factor beta (TGFβ) in IPF patient-derived lung fibroblasts [125]. Changes in cross-linking may have significant impact on the detection of collagens in MS experiments due to the genera-
tion of cross-linked peptides that are missed during the data analysis step. In addition, cross-linking decreases trypsin acces-
sibility to cleavage sites resulting in larger peptides that may require an increase in the range of the m/z window when acquiring MS data. More recently, new quantitative methodo-
logies for the measurement of lysyl oxidase (LOX)-mediated cross-linking of collagen by MS have been developed to address some of the shortcomings of conventional MS analysis [126]. This method has helped to reveal the role of tumor-
associated macrophages in collagen cross-linking and promo-
tion of breast cancer progression.

Recent developments in glycomic and glycoproteomic ana-
lysis of matrissome components can help with the character-
ization and quantification of glycopeptides. For instance, the Zaia laboratory has developed a method based on the enrich-
ment of glycopeptides by retention on a 10kDa molecular weight cutoff filter. Retained glycopeptides are digested by chondroitinase and analyzed by MS which allowed for the identification and assignment of glycosites in a number of proteoglycans such as decorin, brevican, or aggrecan [127]. Furthermore, Merl-Pham et al. has previously used MS to identify fibrosis-related differences in hydroxylation and gly-
cosylation profiles of collagens in IPF patient derived fibro-
blasts [125]. It is also known that pathophysiological processes such as fibrosis are also associated with alterations in the expression and activity of ECM degrading enzymes [128]. Proteomic approaches such as Terminal Amine Isotopic Labeling of Substrates (TAILS) [129] or Proteolytic Signature Peptides (PSP) [130] with subsequent bioinformatic processing originally developed for degradomic studies can provide a powerful tool for detailed analysis of ECM remodeling. We anticipate that future developments in the area of PTMs and matrissome degradation will provide a more holistic and com-
prehensive overview of the complex role of the matrissome in pathophysiology.

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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