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Photocleavable Surfactant-Enabled Extracellular Matrix Proteomics

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Abstract

The extracellular matrix (ECM) provides an architectural meshwork that surrounds and supports cells. The dysregulation of heavily post-translationally modified ECM proteins directly contributes to various diseases. Mass spectrometry (MS)-based proteomics is an ideal tool to identify ECM proteins and characterize their post-translational modifications, but ECM proteomics remains extremely challenging owing to the extremely low solubility of the ECM. Herein, enabled by effective solubilization of ECM proteins using our recently developed photocleavable surfactant, Azo, we have developed a streamlined ECM proteomic strategy that allows fast tissue decellularization, efficient extraction and enrichment of ECM proteins, and rapid digestion prior to reversed-phase liquid chromatography (RPLC)-MS analysis. A total of 173 and 225 unique ECM proteins from mouse mammary tumors have been identified using 1D and 2D RPLC-MS/MS, respectively. Moreover, 87 (from 1DLC-MS/MS) and 229 (from 2DLC-MS/MS) post-translational modifications of ECM proteins, including glycosylation, phosphorylation, and hydroxylation,

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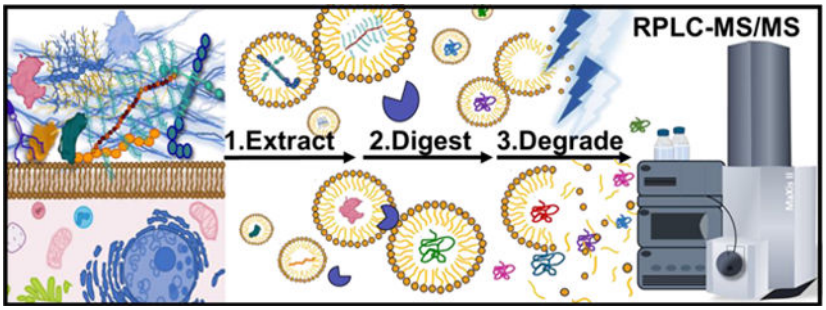
SUPPORTING INFORMATION

The Supplemental Materials and Methods are available free of charge on the ACS Publications website at Chemicals and materials, Mouse mammary tumor tissue; ECM protein extraction; Digestion and 1D-RPLC-MS/MS; SDS-PAGE; Offline high-pH reverse-phase fractionation; Data analysis; Azo enabled two-step extraction for analysis of extracellular matrix (ECM) proteins (Figure S1); Pictures were taken after tissue decellularization and washing steps (Figure S2); 8% SDS-PAGE analysis of representative ECM extraction replicates of Decell extract 1 and Azo extract 2 (Figure S3); Scatter-plots of Log2 transformed relative ECM protein abundances in extraction replicates of Azo extract 2 from mammary mouse tumor tissue (Figure S4); Composition of Decell extract 1 and Azo extract 2 from mammary mouse tumor tissue (Figure S5); Comparison of Azo to the traditional ECM extraction buffers (Figure S6); Comparison of 1- and 2-dimensional LC separation strategies for ECM proteomics (Figure S7); Summary of PTM sites identified in ECM proteins from mammary mouse tumor tissue (Figure S8); Data analysis parameters (Table S1); Inventory of ECM protein identifications (Table S2); Summary of label-free quantitation of ECM proteins (Table S3); Summary of post-translational modifications of ECM proteins (Table S4).

were identified and localized. This Azo-enabled ECM proteomics strategy will streamline the analysis of ECM proteins and promote the study of ECM biology.

Graphical Abstract



Keywords

Extracellular matrix; matrisome; photocleavable surfactant; post-translational modification; glycoproteins

The extracellular matrix (ECM) consists of approximately 300 core proteins including collagens, fibronectins, laminins, and proteoglycans and ~900 associated proteins, defined as the "matrisome"[1–2], which forms an architectural meshwork and provides stability for the surrounding cells.[1–5] Serving as a critical regulator of cell behaviors such as adhesion, migration, and proliferation, the ECM responds and communicates via biochemical cues to the intracellular milieu.[3] ECM proteins are also known to be heavily post-translationally modified, most notably by glycosylation and hydroxylation.[6,7] Dysregulation of ECM protein expression and post-translational modifications (PTMs) directly contribute to disease progression and regulate ECM protein structures, functions, and interactions contributing to pathogenesis.[1–10] Particularly, the ECM is increasingly recognized as a major driver in tumor metastasis, which contributes to 90% of the cancer deaths.[11–13] However, the biochemical characterization of ECM proteins remains a daunting task due to the extremely low solubility of ECM proteins.[14–16]

Advances in mass spectrometry (MS)-based proteomics make it an ideal tool to identify ECM proteins and characterize their PTMs occurring within the ECM microenvironment.[2, 7, 17–28] Despite its immense potential, ECM proteomics remains extremely challenging mainly due to the dense network of cross-linked, fibrous proteins, which makes it exceedingly difficult to solubilize, digest, and analyze by MS.[19] Moreover, the complexity and dynamic range of proteins present in the tissue lysates often result in underrepresentation of the important ECM sub-proteome in global proteomics studies.[29] The current protocols for ECM proteomic analysis typically have lengthy and labor-intensive workflows that often include multiple digestion steps, some including chemical digestion using toxic chemicals such as cyanogen bromide (CNBr).[20, 21, 30]

To address these challenges, here we developed a photocleavable surfactant-enabled ECM proteomics strategy to streamline the enrichment, extraction, and digestion steps for ECM

proteomics (Figure 1). Specifically, we established a new decellularization/extraction method, enabled by our recently developed photocleavable anionic surfactant, Azo,[31, 32] for the efficient enrichment of ECM proteins, which eliminates the need for multiple digestion steps and minimizes the sample clean-up prior to reversed phased liquid chromatography (RPLC)-MS analysis. Our sample preparation method takes approximately ~8 h (0.75 h for decellularization, 1.5 h for removal of Triton and work-up of the Decell extract, 1.5 h for extraction of the pellet, and 3.5 h for reduction, alkylation and trypsin digestion) compared to the conventional approaches that often take several days due to lengthy decellularization and multiple enzymatic digestions,[20, 33] representing a significant improvement in the throughput of ECM proteomics. Specifically, we chose to analyze tumor tissues harvested from the transgenic mouse mammary tumor virus (MMTV) polyomavirus middle T (PyVT) mouse model as it represents a classic transgenic model for studying the microenvironment of metastatic human breast cancer[34–37] and extensive collagen deposition is a pathological hallmark of many cancers.[38, 39]

RESULTS AND DISCUSSION

For characterization of the mouse tumor tissue, we considered both the core ECM domain-containing regions as well as regulatory and secreted ECM associated proteins, which constitute “the matrisome” as defined previously.[1, 2] The core matrisome was classified into three subcategories, collagens, glycoproteins, and proteoglycans, whereas regulators, secreted factors, and affiliated proteins were classified as associated matrisome proteins (Figure 2a). Characterizing the ensemble of core and associated matrisome proteins is critical to achieving a comprehensive understanding of ECM biology.[1, 2] However, core matrisome proteins, like collagens, are generally considered extremely challenging to be analyzed by proteomics since they are extremely difficult to be solubilized,[40] and resistant to common extraction and enzymatic digestions.[41]

Typically, enrichment of the core matrisome proteins is obtained by decellularization,[42] wherein the tissue is passively immersed in a buffer, such as SDS or Triton X-100, for several hours or days to remove soluble cellular material (proteins, lipids, metabolites, etc.), leaving an intact ECM.[20, 21, 43] However, since an intact scaffold is not required for proteomics, we mechanically homogenized the tissue in Triton X-100 buffer[44] to dramatically increase the throughput. The samples were centrifuged and the supernatant was collected and labeled as “Decell extract 1”, and a pellet remained (Figure S1). Commonly, proteins extracted during decellularization are discarded[20] presumably because few ECM proteins are extracted during this step. To investigate whether the decellularization extraction contains soluble ECM factors, the proteins in the Decell extract 1 were precipitated and reconstituted in 0.5% Azo for downstream LC-MS analysis (Figure S1).

Next, the remaining tissue pellets were washed with ammonium bicarbonate (ABC) buffer (chosen for its MS-compatibility) to remove any remaining soluble proteins, surfactants, or salts (Figure S2). Azo was then used to extract the remaining proteins yielding “Azo extract 2” for further LC-MS analysis. We observed excellent reproducibility using SDS-PAGE analysis across technical replicates (Figure S3). Interestingly, we qualitatively observed that

Decell extract 1 was rich in lower molecular weight proteins, whereas Azo extract 2 contained many higher molecular weight species (Figure S3).

Subsequently, Decell extract 1 and Azo extract 2 were digested in-solution with trypsin for 2 h followed by rapid degradation of Azo[32] by UV light and subsequent analysis by RPLC-MS/MS. Recently we developed a high-throughput bottom-up proteomics method enabled by this photocleavable surfactant for robust protein extraction, rapid enzymatic digestion, and subsequent MS-analysis without additional sample clean-up following UV degradation.[32] Identification and relative protein abundance including reproducibility across replicates were determined with MetaMorpheus[45] and FlashLFQ[46] software using intensity-based normalization. A full list of parameters can be found in Table S1 and a full list of ECM identifications can be found in Table S2. Transformed, normalized intensities of ECM peptide spectral matches (PSMs) in Azo extract 2 were plotted against each other, demonstrating a highly reproducible method with Pearson correlation coefficients from 0.91 to 0.93 (Figure S4).

Using the normalized peptide intensities, we analyzed the relative abundance of core and associated matrisome proteins in the Decell and Azo extracts (Figure S5a and Table S3). Significantly, we observed the Azo extract 2 generally contained a higher abundance of core matrisome proteins such as collagen alpha-1 (I) chain (COL1A1) and biglycan (BGN) (Figure S5b–c and Table S3). On the other hand, associated matrisome proteins, such as Galectin-1 (LGALS1) and Cathepsin D (CTSD), were enriched in the Decell extract 1 (Figure S5b–c) (Table S3). Additionally, we evaluated the cellular locations of proteins in both extractions to better understand their protein compositions (Figure S5a). Endoplasmic reticulum (ER), mitochondrial, cytoplasmic, secreted and membrane proteins were found in higher abundance in the Decell extract 1, whereas core matrisome and nuclear proteins were primarily present in Azo extract 2 (Figure S5c–d). Overall, the results demonstrated successful protein fractionation at the extraction level and highlighted Azo's ability to solubilize and digest important ECM proteins such as type I collagen[47] for a streamlined analysis.

To further assess Azo's extraction efficacy, we directly compared its performance to 8 M urea, a common reagent used for ECM protein extraction.[31,32,25] Pulverized tumor tissue was decellularized with Triton-X-100, washed, and extracted with 8 M urea in 25 mM ABC, 0.5 % Azo in 25 mM ABC, or 25 mM ABC (serving as a control). SDS-PAGE analysis demonstrated Azo extracted a unique protein profile compared to the other conditions (Figure S6a). Using RPLC-MS/MS analysis, we found the relative abundance of collagen species, a common benchmark for ECM enrichment,[2, 14] was significantly increased in the Azo extract compared to the urea or ABC alone (Figure S6b and Table S3). Hence, this demonstrated the superior performance of Azo relative to urea, in solubilizing ECM proteins. In particular, fibrillar type I collagen chains, COL1A1 AND COL1A2, were solubilized more efficiently with Azo relative to urea extraction. Moreover, the use of photocleavable Azo eliminated the need for time-consuming desalting steps and greatly improved the throughput of ECM proteomics. Overall, we identified 173 ECM proteins using 1D RPLC-MS/MS. A total of 52 and 71 proteins were uniquely identified in the Decell extract 1 and Azo extract 2 samples, respectively, and 50 proteins were identified in

both (Figure S7a). Next, we investigated whether the addition of protein fractionation by high-pH RPLC could increase the proteome coverage. This 2D high-pH low-pH RPLC approach contributed 52 new unique identifications (a 30% increase) compared to using 1D RPLC-MS/MS (Figure S7a–b). Although the increase in protein identification was moderate, we demonstrated our method could be easily combined with additional separation steps to increase proteome coverage.

Combined, we identified 225 unique ECM proteins from mouse mammary tumor tissue using this approach (Figure 2b and Table S2). Importantly, we observed both Decell extract 1 and Azo extract 2 contained many matrisome proteins. Of the ECM protein identifications, 89 ECM proteins were identified in both extracts, whereas 51 and 85 were uniquely identified in Decell extract 1 and Azo extract 2, respectively, which indicates that some proteins are preferentially solubilized and present in either Decell extract 1 or Azo extract 2 (Figure 2b).

We further investigated the protein compositions of each extract, observing that 54 core matrisome proteins and 86 associated proteins were identified in the Decell extract 1 whereas 96 core matrisome proteins and 63 associated proteins were identified in Azo extract 2 (Figure 2c). The results indicate enrichment of core matrisome proteins in the Azo extract 2, but also highlight the importance of analyzing the commonly disregarded decellularization fraction, Decell extract 1. Additionally, Decell extract 1 and Azo extract 2 contained similar numbers of proteoglycans, secreted proteins, and affiliated proteins (Figure 2d). On the other hand, ECM regulatory proteins were enriched in the Decell extract 1 whereas a large number of collagens and glycoproteins were identified in the Azo extract 2. All categories of ECM proteins were well represented, using our approach (Figure 2e). We observed that a larger number of higher molecular weight (>100 kDa) and core matrisome proteins were identified in Azo extract 2, whereas a larger number of lower molecular weight, associated matrisome species were identified in Decell extract 1 (Figure 2f). We reason that the higher molecular weight of core matrisome proteins makes them more difficult to be solubilized, thus require a strong surfactant such as Azo. A representative list of identified ECM proteins is shown in Figure 2g.

ECM proteins are known to be heavily post-translationally modified. Here, we used MetaMorpheus[45] software to identify a diverse range of ECM PTMs including proline hydroxylation, asparagine hydroxylation, lysine hydroxylation, acetylation, phosphorylation and glycosylation (Figure S8). In total, 87 unique PTM sites were identified from mouse tumor tissue using 1D RPLC-MS/MS (Figure S8 and Table S4). Significantly, we observed a dramatic increase in PTM identifications, 229 total PTMs, with the 2DLC-MS/MS workflow (Figure S8 and Table S4), despite that the 2D approach provided only a moderate increase in the identifications of ECM proteins. Overall, proline hydroxylation, which is essential for the stability of collagen fibrils,[47] accounted for 50% of all identified modifications from mouse mammary tumor tissue (Figure S8). Figure 3 shows some representative examples of unambiguously localized PTMs, such as proline hydroxylation of collagen alpha-2 (I) chain (COL1A2) and asparagine β hydroxylation, on proteins like fibrillin-1 (FBN1) (Figure 3a–b). N-acetylglucosamine (HexNAc) was localized to a threonine residue of Host cell-factor-1

(Figure 3c). Additionally, we have identified an N-terminal acetylation of Serpin B6 (Figure 3d).

CONCLUSION

In summary, for the first time, we developed a streamlined ECM proteomics method enabled by a photocleavable surfactant, Azo, which addressed several challenges in the conventional ECM proteomics workflows. Most notably, Azo facilitated robust extraction of fibrous ECM proteins, aided in rapid trypsin digestion, and subsequently can be easily degraded by UV-irradiation before MS. This Azo-enabled ECM sample preparation including decellularization, ECM extraction, reduction, alkylation and digestion takes about 6 h compared to several days or a week using the conventional approaches. This sample preparation does not require chemical digestion, multiple enzymes, or deglycosylation. Moreover, we demonstrated the importance of analyzing the commonly discarded decellularization fraction to identify soluble ECM and ECM-associated proteins. Using both 1D and 2D RPLC-MS/MS, we have established an ECM protein catalog consisting of 225 ECM proteins from mouse tumor tissue and have further identified 229 total PTMs for ECM proteins including hydroxylation, phosphorylation, and glycosylation. We envision this Azo-enabled ECM proteomics strategy will streamline the analysis of ECM proteins and promote an understanding of ECM biology in various diseases such as tumor metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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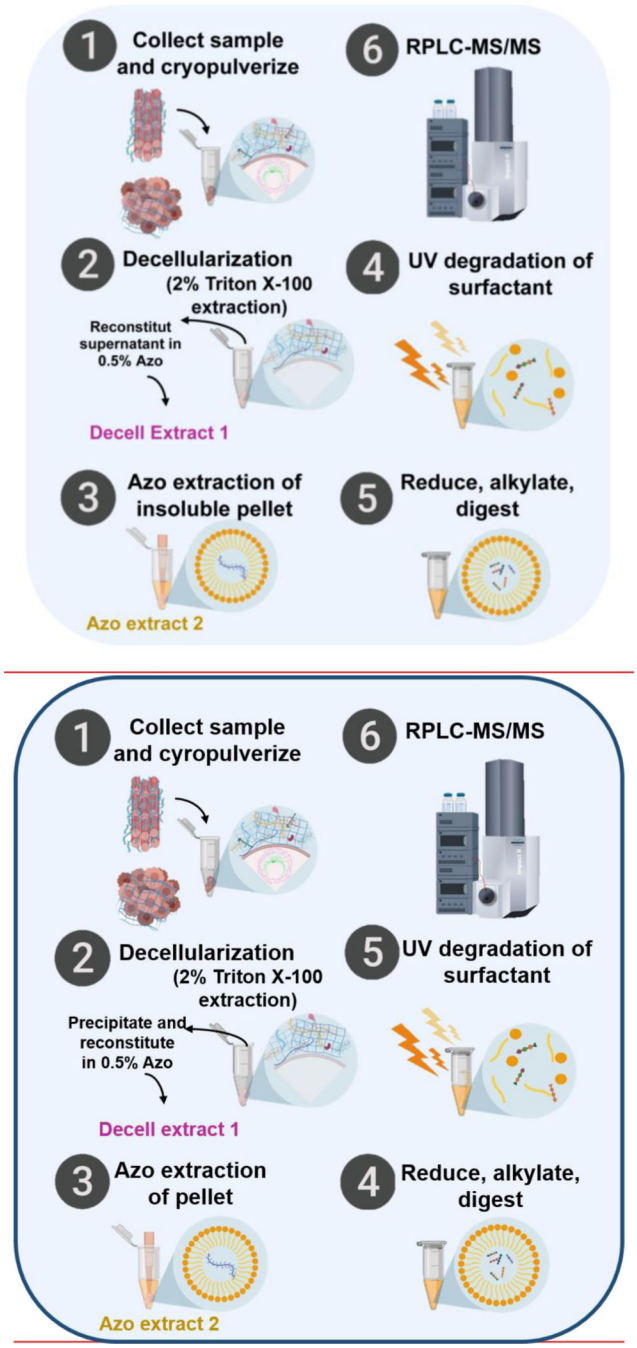
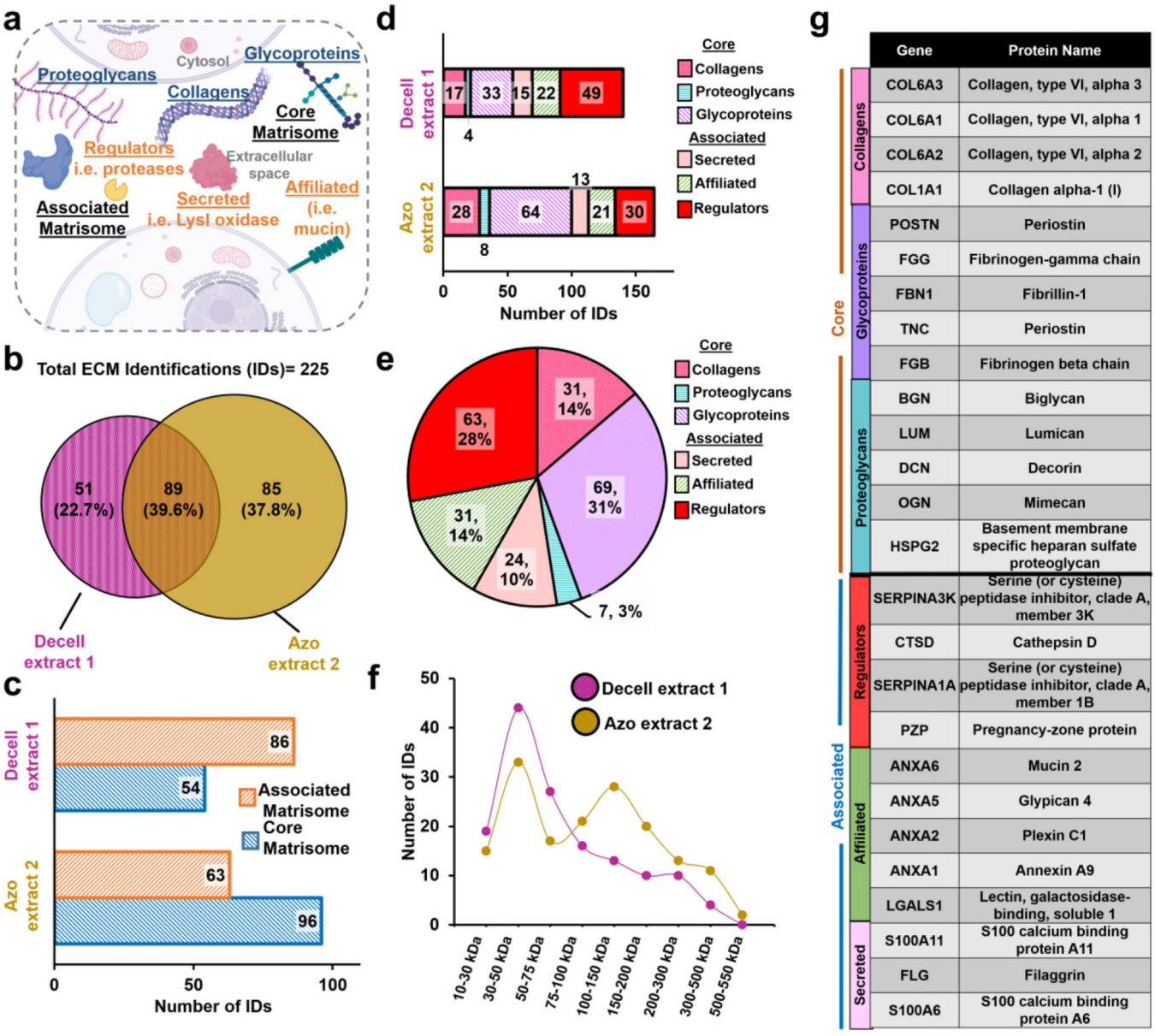


Figure 1.

Schematic illustration of the Azo-enabled ECM proteomics strategy. (1) Tissues are collected, cryo-pulverized, and (2) undergo rapid decellularization in 2% Triton X-100. After centrifugation, the supernatants are collected, precipitated, and reconstituted in 0.5% Azo labeled as “Decell extract 1” for downstream analysis. (3) Azo was added to the remaining tissue pellet to extract the remaining proteins, resulting in “Azo extract 2”. (4) Both Decell Extract 1 and Azo extract 2 were reduced, alkylated, and digested with trypsin.

(5) Azo was degraded by UV-irradiation and (6) the digested peptides were analyzed by RPLC-MS/MS.



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	Gene	Protein Name
Core / Collagens	COL6A3	Collagen, type VI, alpha 3
	COL6A1	Collagen, type VI, alpha 1
	COL6A2	Collagen, type VI, alpha 2
	COL1A1	Collagen alpha-1 (I)
Core / Glycoproteins	POSTN	Periostin
	FGG	Fibrinogen-gamma chain
	FBN1	Fibrillin-1
	TNC	Periostin
	FGB	Fibrinogen beta chain
Core / Proteoglycans	BGN	Biglycan
	LUM	Lumican
	DCN	Decorin
	OGN	Mimecan
	HSPG2	Basement membrane specific heparan sulfate proteoglycan
Associated / Regulators	SERPINA3K	Serine (or cysteine) peptidase inhibitor, clade A, member 3K
	CTSD	Cathepsin D
	SERPINA1A	Serine (or cysteine) peptidase inhibitor, clade A, member 1B
	PZP	Pregnancy-zone protein
Associated / Affiliated	ANXA6	Mucin 2
	ANXA5	Glypican 4
	ANXA2	Plexin C1
	ANXA1	Annexin A9
	LGALS1	Lectin, galactosidase-binding, soluble 1
Associated / Secreted	S100A11	S100 calcium binding protein A11
	FLG	Filaggrin
	S100A6	S100 calcium binding protein A6

Figure 2.

ECM proteomics of mammary tumor tissue. **a.** Schematic of matrisome ECM composition and annotation.[1, 2] **b.** Venn diagrams showing the overlap of ECM protein identifications found in the Decell extract 1 and Azo extract 2 identified using 2D RPLC MSMS. **c.** Division distribution of ECM identifications in each extract. **d.** Category distribution of ECM identifications in each extract. **e.** Pie chart illustrating the composition of all ECM identifications. **f.** The molecular weight (MW) distribution of all ECM identifications. **g.** Table of highest abundance ECM core matrisome proteins identified in Azo extract and associated matrisome proteins identified in Decell extract 1.

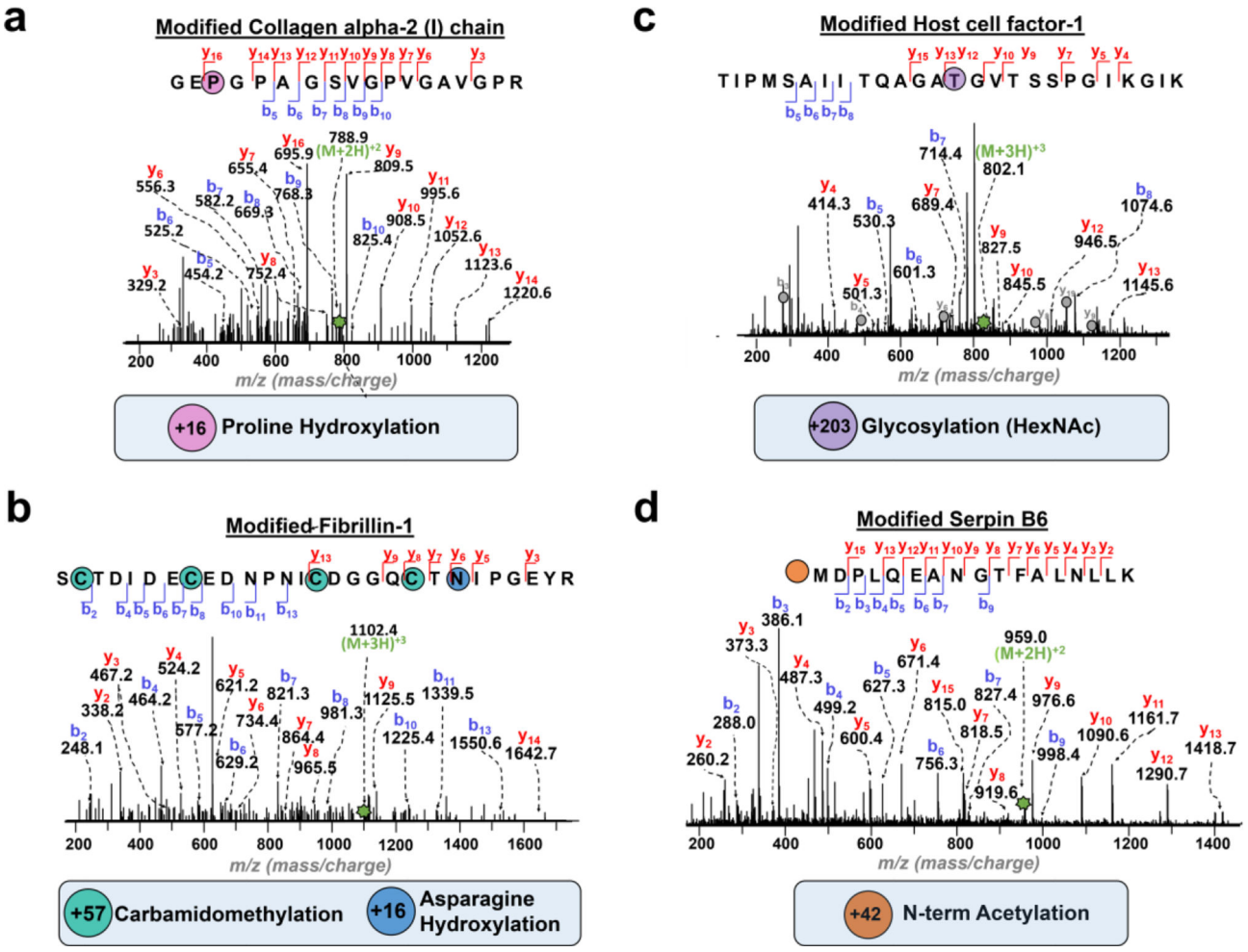

Figure 3. Identification of PTMs in the ECM proteins.

Representative MS/MS spectra of identified ECM PTMs. Precursor ions are annotated with green stars. **a.** Precursor ion Collagen alpha-2 (I) chain peptide with proline (P) hydroxylation (+ 16 Da). [$(M+2H)^{2+}$, 788.9 m/z, Expt'l: 1,575.8 Da, 0.8 ppm]. **b.** Fibrillin-1 peptide modified with cysteine (C) carbamidomethylation (+ 57 Da) and asparagine (N) hydroxylation (+16 Da). Precursor ion [$(M+3H)^{3+}$, 1102.4 m/z, Expt'l: 3,304.3 Da, 1.4 ppm]. **c.** Host cell-factor-1 with (N)-linked acetylhexosamine (HexNAc) glycosylation (+203 Da). Precursor ion [$(M+3H)^{3+}$, 802.1 m/z, Expt'l: 2,403.3 Da, 0.6 ppm]. HCFC1 co-eluted with a highly abundant peptide identified as splicing factor 3b subunit 2, where corresponding b and y ions are denoted with gray circles. **d.** Serpin B6 with N-terminal acetylation (+42 Da). Precursor ion [$(M+2H)^{2+}$, 959.0 m/z, Expt'l: 1,916.0 Da, 0.2 ppm].