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Proteomic analysis of the cardiac extracellular matrix: clinical research applications

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Abstract

Introduction—The cardiac extracellular matrix (ECM) provides anatomical, biochemical, and physiological support to the left ventricle. ECM proteins are difficult to detect using unbiased proteomic approaches due to solubility issues and a relatively low abundance compared to cytoplasmic and mitochondrial proteins present in highly prevalent cardiomyocytes.

Areas covered—Proteomic capabilities have dramatically improved over the past 20 years, due to enhanced sample preparation protocols and increased capabilities in mass spectrometry (MS), database searching, and bioinformatics analysis. This review summarizes technological advancements made in proteomic applications that make ECM proteomics highly feasible.

Expert commentary—Proteomic analysis of the ECM provides an important contribution to our understanding of the molecular and cellular processes associated with cardiovascular disease. Using results generated from proteomics approaches in basic science applications and integrating proteomics templates into clinical research protocols will aid in efforts to personalize medicine.

Keywords

Extracellular matrix; cardiac remodeling; proteomics; cardiovascular disease

1. Introduction

The cardiac extracellular matrix (ECM) provides structural and biochemical support for the cell types that comprise the myocardium, including cardiomyocytes, endothelial cells, and vascular smooth muscle cells, fibroblasts, and immune cells [1,2]. Because ECM is by nature insoluble and present in relatively low abundance compared to more prevalent

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Declaration of interest

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cytoplasmic and mitochondrial proteins found as cell constituents, ECM proteins are often not detected during global proteomics analyses using unbiased approaches [3].

The cardiac ECM consists of a complex network of fibrillar proteins (predominantly collagen type I and type III), elastic fibers, glycosaminoglycans, glycoproteins, and adhesive proteoglycans [4]. ECM proteins support physiology of the normal myocardium and regulate the extent of fibrosis and impaired physiology that occurs during disease progression. After myocardial infarction (MI), for example, the myocardium undergoes extensive protein turnover as old ECM is replaced by an infarct scar primarily composed of collagen [2,5]. Cardiac wound healing is dependent on a balance between ECM removal and synthesis of new ECM. Excess degradation by matrix metalloproteinases (MMPs) can lead to aneurysms or rupture of the left ventricle (LV) whereas excess deposition leads to a stiff, noncompliant LV and the development of heart failure [2,5–8]. For these reasons, assessing cardiac ECM for early predictors of adverse remodeling may reveal targets for novel therapies.

Proteomics is the large-scale study of all proteins present in the sample at the time of evaluation and encompasses several layers of complexity [9]. Proteomics has evolved over the past 20 years, moving from primarily observational studies to studies that couple with cell or tissue physiological assessments. Observational proteomics describes experiments that catalog and quantify protein changes in samples, whereas functional or physiological proteomics describes experiments that determine protein quality, evaluate protein function, and explore protein interactions.

Several reviews have emerged recently focused specifically on cardiovascular proteomics. Reviews from the Lindsey and Mayr labs have focused on the cardiovascular ECM, and the Hynes' lab has focused on cataloging ECM proteomes in multiple tissues including ovarian and breast cancer [1,2,10–13]. This review summarizes how the ECM proteomics field has evolved, including enhanced sample preparation protocols to increase representation of ECM proteins, coupled to improved mass spectrometry (MS), database searching, and bioinformatics analysis capabilities. Combined, these improvements make evaluation of the cardiac ECM practical [2,4,14–16]. We will also discuss current limitations and ways to improve to take full advantage of current technologies.

2. Proteomics challenges with ECM

Physiological ECM proteomics is complex and a necessary component in the goal to dissect the critical mechanistic roles of LV ECM remodeling [2,4,14,17]. ECM proteins are present in relatively low abundance when compared to cytosolic, myofilament, or mitochondrial components and form complex macromolecular structures with low solubility in aqueous solutions [2,14]. This can make identification and quantification of ECM proteins difficult. Below we discuss the tools our lab and others have developed to enrich for ECM and evaluate plasma and tissue samples from human subjects following MI (Table 1).

2.1. Indexing ECM proteins expressed in the LV

Natural enrichment strategy—In the setting of MI, focusing on the infarct region in the acute setting is important, as the infarct is a metabolically active region of intense ECM

turnover. Focusing on the remote region predisposes one to examine easily soluble mitochondrial and cytoplasmic proteins, which are highly abundant in this region. Many of the changes in myocyte proteins are a consequence of the MI and not necessarily relevant to the wound healing response. A natural enrichment strategy of isolating the infarct region takes advantage of the overabundance of collagen and other ECM proteins in the newly formed scar. By picrosirius red histological staining, the day 7 infarct scar is comprised of 30% collagen, indicating a large amount of ECM enrichment at this time point due to the cardiac wound healing process [18,19]. Taking advantage of the fact that day 7 infarcts have a large ECM composition, we used two dimensional (2D)-gel electrophoresis coupled to MS to identify a number of non-collagen ECM proteins, including fibronectin, laminin, peroxiredoxin-1, -2, and -3, tenascin C, and thrombospondin 1 [20,21]. Using mice with global deletion for MMP-7 or MMP-9 genes compared to wild-type (WT) mice, we classified a number of new MMP substrates [17,20,21]. As an example, we demonstrated *in vivo* that fibronectin was an infarct-relevant substrate. While this approach proved to be a step above previous attempts using whole myocardial samples, there was still a large amount of intracellular proteins in the samples.

Decellularization and differential solubility-based approaches—Tissue decellularization is another method we and others have used to enrich for ECM. Decellularization has been used to assess ECM composition in human vascular samples, including samples from human abdominal aneurysms and symptomatic carotid plaques [12,22–24]. The decellularization process primarily involves incubating samples in 1% sodium dodecyl sulfate (SDS). SDS affords the dual advantage of efficiently extracting proteins and enhancing protein solubility by effectively permeabilizing the cell membrane to remove cellular components [3]. The Mayr laboratory has also developed a sequential extraction methodology to increase solubility of cardiac ECM [24]. In their protocol, tissue first undergoes decellularization followed by re-solubilization and centrifugation to fractionate protein according to solubility.

The use of decellurization has increased the ability to qualify ECM proteins of interest that are usually masked by highly abundant proteins. Mayr et al. used this approach to identify novel ECM proteins that contribute to cardiac remodeling in a porcine model of ischemia/reperfusion [23]. Biosignatures derived from proteomics experiments can be used to indicate insufficient or overly robust post-MI wound healing and predict patients at risk for heart failure development.

Our team developed the Texas 3-step decellurization approach [3]. This approach incorporated three sequential separation steps to reduce complexity by dividing samples into three fractions: soluble, cellular, and insoluble proteins. In this approach, samples are first incubated in a neutral low salt, non-denaturing buffer (0.5 M NaCl, 10 mM Tris base pH 7.5, and 1× PI) for extraction of soluble proteins (Step 1). This includes newly synthesized ECM proteins and degradation products. The remaining tissue undergoes decellularization by treatment with SDS (Step 2), and finally, the insoluble protein fraction is exposed to acid extraction, deglycosylation, and re-solubilization (Step 3). While the Texas 3-step was good for providing an unbiased first pass to identify novel proteins of interest, myocardial samples showed large variation due to the multiple extraction steps, making quantitative comparisons

between groups difficult. Decellularization pushed the ECM proteomic field forward primarily by providing a first pass ECM catalog that included many proteins not previously evaluated in the myocardium.

Hydrazide bead glycoprotein enrichment—The Zhang and Nilsson labs pioneered the field of glycoproteomics using a hydrazide chemistry-based approach to isolate glycoproteins and glycopeptides [25,26]. Targeting glycosylated proteins provides the capability to identify membrane and extracellular proteins that may be difficult to detect otherwise. Glycoproteomics is highly efficient and can be easily incorporated into a proteomics workflow for integrated analysis. Nilsson et al. extended this approach to characterize intact sialylated glycopeptides by covalently binding sialylated glycoproteins to hydrazide beads which were then released by acid hydrolysis [25,27].

Using hydrazide beads to enrich for glycoproteins has been successfully applied to the quantitative analysis of complex mixtures, including plasma [26,28–34]. Our team has recently employed gel-free solid-phase extraction of N-linked glycopeptides (SPEG) to enrich for cardiac ECM proteins in both plasma and tissue samples [31,32,35]. Using this approach, we have identified a number of MMP substrates with roles in LV remodeling. For example, out of 541 N-glycosylated proteins quantified, we identified CD36 as a novel MMP-9 substrate in infarct tissue of mice. Degradation of CD36 by MMP-9 led to decreased macrophage phagocytosis and prolonged neutrophil survival in the MI setting [32]. A word of caution with the SPEG method, as quantification of glycosylated protein may not reflect total protein amounts if there is a large amount of non-glycosylated protein in the mix. Secondary validation by an independent technique such as multiple reaction monitoring, ELISA, or immunoblotting is needed to determine total protein concentrations [36,37].

One limitation of hydrazide chemistry is this technique is limited to N-linked glycoprotein analysis due to the lack of an efficient approach to cleave O-linked glycoproteins and glycopeptides [22]. Furthermore, this enrichment approach identifies deglycosylated glycoproteins and glycopeptides, which may or may not actually be glycosylated *in vivo*. In addition, the availability of N-glycosites per protein is minimal, which can make identifying the corresponding protein challenging [28].

2.2. Indexing ECM proteins expressed in plasma

Several large-scale proteomics studies have been undertaken to determine the glycoproteomic profile of human plasma and serum [26,33,35,38–40]. Similar to tissue proteomics, plasma proteomics has long been burdened by technical issues. The 10 most abundant proteins account for 90% of total protein concentration, making it difficult to identify novel proteins of interest [41–43]. One abundant protein, serum albumin, often has to be removed prior to proteomics analysis [43]. There are several commercially available albumin removal kits; most of which are based in immunoaffinity columns. Albumin can also be removed by ligand chromatography and isoelectric trapping [44–46]. Our group has utilized a gel-free plasma fractionation technique using the GelFree 8100 Fractionation System by Protein Discovery, Inc. prior to proteomics analysis. Using this technique, we were able to reduce the presence of highly abundant proteins and enrich samples for the

lower abundant ECM proteins [43]. One issue with albumin depletion is this may also result in removal of low abundant cytokines, lipoproteins, and ECM proteins of interest, especially if these proteins are bound to albumin [16].

Several highly abundant proteins found in the plasma, including albumin, do not contain N-glycosites allowing for more efficient identification and quantification of the lower abundance glycoproteins in circulation [30]. Using the SPEG approach, we enriched for ECM protein in plasma, as 74% of the peptides mapped to the extracellular compartment [35]. The de Castro Bras lab identified a novel cardiac collagen I matricryptin that was reduced in the plasma of post-MI patients compared to healthy controls [17]. They found that this matricryptin facilitated LV remodeling post-MI by regulating scar formation through targeted ECM generation and stimulation of angiogenesis. This study highlights how cardiac ECM proteins can leak into circulation during disease facilitating with diagnosis of adverse cardiac remodeling and disease progression.

2.3. Techniques used for quantification of ECM proteins

Consistency and accuracy of MS-based proteomics depends on the performance capabilities of the instruments, acquisition methods, and analysis software. Quantification can be achieved using both label and label-free approaches [47,48]. Proteomic data are multiplexed to target many peptides in a single assay increasing throughput.

Label-free quantification—Label-free quantification is based on the integrated peptide peak intensities. Peptide signals are detected and distinguished from chemical noise by the peptides isotopic pattern. The total ion current of the peptide signal is then integrated and used as a quantitative measurement of the original peptide concentration. We have used label-free proteomics to identify plasma proteins differentially expressed in WT and MMP-9 null mice [49].

The Mayr laboratory used a label-free approach to quantify the secretome of human endothelial cells. This study provided the most comprehensive catalog of endothelial protein secretion to date [10]. Secretomics is the detection of proteins secreted or shed into the culture medium by cells. Secretomics has been used to evaluate miRNA targets that were involved in fibrosis, including collagen, MMPs, leukemia inhibitory factor, insulin-like growth factor 1, and pentraxin 3 [50].

Isotopic labeling—Another approach for MS quantification involves labeling the samples with isobaric tags, such as those used for relative and absolute quantitation (iTRAQ) or tandem mass tag (TMT) [51]. This allows the mass spectrometer to distinguish between identical proteins in separate samples [52]. While multiplexing can be costly and sample processing time consuming, overall this technique reduces running time and is less affected by experimental bias than label-free quantification. In addition, labeling is introduced after peptide digestion, and therefore, isobaric tags cannot evaluate *in vivo* or *in vitro* protein changes but only for quantitative comparisons using tissue samples [11].

Unlike isobaric tags, stable isotope labeling with amino acids in cell culture (SILAC) allows for *in vivo* and *in vitro* labeling through metabolic incorporation of stable isotope analogs

into all newly synthesized proteins. SILAC has been used for quantitative comparisons of newly synthesized ECM proteins in human mesenchymal stem cells [53]. In addition, this strategy has been used to determine that the left and right ventricle proteomes are very similar qualitatively as well as quantitatively under non-pathological conditions [54].

SILAC can also be used to quantify protein complexes, enzyme substrates, membrane proteins, and temporal dynamics [55–57]. Pinto et al. demonstrated the utility of SILAC for exploring sheddase activity in cultured cells to identify two novel substrates for snake venom metalloproteinases [58,59]. A limitation of using SILAC is that the cells must be cultured in protein-free medium to allow analysis of secreted proteins in the supernatant; thus, using this approach with post-mitotic cells such as cardiomyocytes is limiting. In addition, using serum-free media conditions assumes proteins secreted during starvation reflect *in vivo* secretion patterns, an assumption which may not be true.

Targeting proteomics—There are three main approaches for targeted proteomics: selected reaction monitoring (SRM) [60], parallel reaction monitoring (PRM) [61,62], and data-independent acquisition (DIA) [63,64]. One benefit to using techniques such as SRM and PRM is that quantification of target proteins across many samples is highly accurate and reproducible. Studies have shown strong correlation between targeted proteomic techniques like SRM and ELISA biomarker assays in patients with MI [65]. However, the multiplexing capability is confined to typically ~500 peptides/125 proteins per analysis in order to ensure reliable quantification [60,62]. Increasing the number of target peptides would decrease the sensitivity, especially for PRM whose sensitivity is inversely proportional to the degree of multiplexing [66].

DIA-based targeted quantification (e.g. SWATH) was developed for proteome-wide quantification of target proteins of interest in order to alleviate the limitations in multiplexing [63,64]. DIA is highly reproducible and accurate due to its unbiased, broad range of precursor ion selection and fragmentation. Even so, DIA analysis can be challenging due to the resulting fragment ion spectra being highly multiplexed. This can introduce noise and reduces precursor selectivity five-to tenfold compared to data obtained with SRM [67]. To overcome these issues, a few software tools have been developed such as OpenSWATH [68]. Overall, targeted proteomics hold promise in advancing our understanding of protein networks and their pathological significance. Development of these techniques will aid in the advancement of biomarkers into clinical use.

2.4. Qualitative characterization of ECM

In addition to traditional methods for evaluation of membrane topology, proteomics can be used to provide information about membrane topography [31]. This includes location of N-glycosylation and proteolytic sites, which are relevant to ECM studies [2]. Assessment for N-glycosylation sites can be used for evaluation of membrane topology [69,70]. Gundry et al. used this method to determine the orientation of transmembrane glycoprotein ZIP14, which was incorrectly assigned in SwissProt and has since been corrected [69]. The Gundry lab also uses this approach to identify novel markers of stem cells [71,72]. Our lab used a similar approach to provide new topologic information on hundreds of membrane and ECM

proteins, including identifying four novel N-glycosites for β 1 Integrin (N212, N406, N481, and N520) in post-MI infarct tissue [31]. In order to identify new protein partners, global approaches for large-scale proteomic profiling should be followed up with other methodologies such as nuclear magnetic resonance. To map protein–protein interactions using MS technology, the intact protein complex must first be affinity purified before digestion and MS analysis [2,73].

3. Applying technology to the clinic

Over the past 20 years, the proteomics field has developed rapidly to significantly impact on development of personalized medicine [4,74]. As an attempt to apply proteomic technologies to the clinical setting, various mass spectrometric methods have been developed. While proteomic technologies are understood at the instrument and analysis level, there are particular challenges with complex methods that have multi-analyte outputs. Recent efforts to improve the reproducibility and work flow have improved our ability to identify novel disease markers in the clinic using proteomics. The primary benefit of the translation of proteomic discoveries to meaningful clinical applications in cardiovascular medicine is that proteomics provides an unbiased evaluation of complex protein mixtures [4].

Much of the current research remains in the discovery phase as proteomics in the clinical setting is mostly used for identification of biomarkers. For example, whole tissue proteomics identified multiple structural proteins, including lumican, as being elevated in hypertrophic LVs [75]. Myocardial tissue biopsy specimens collected from the apex of right and left ventricles in patients with aortic stenosis indicated lumican was linked to fibrosis during the transition to heart failure [76]. MS offers an integrated system for identifying both known and unknown biomarkers [77].

Accurate diagnosis of a patient, treatment selection, and therapeutic monitoring can be assessed by MS due to the sensitivity of current instruments. Further advancements in workflow can also improve the time to results, which can be a limiting factor. Mass spectrometers can identify and quantify proteins, peptide fragments, small molecules, antibodies, metabolites, and lipids [77]. Proteomic techniques that enrich for ECM have been used to identify additional changes facilitating in cardiovascular disease progression. The Mayr lab was the first to report the presence of over a dozen ECM proteins not previously identified in the myocardium [23,24]. Utilizing protein network interaction analysis to assess the protein signatures, Mayr and colleagues defined a signature of early- and late-stage cardiac remodeling with transforming growth factor- β 1 signaling as being pivotal for ischemia and reperfusion induced ECM remodeling [23].

Alternative quantitative methods other than MS such as antibody approaches have demonstrated success in the clinical setting [78–80]. One limitation of the current clinical assays used for quantification of biomarkers is that they generally analyze only a single molecule at a time and can require a relatively large sample volume. Being able to measure multiple analytes in one sample will aid in attempts to more accurately predict risk and assess prognosis in patients with cardiovascular disease [81]. Using MS could significantly

reduce the time required for diagnosis, as multiple biomarkers can be detected from a single sample and in a single test [77,82]. Improvements continue to be made in using proteomics for clinical assessment.

4. Expert commentary

At this juncture, we are closer than ever before to being able to use proteomics to capture the entire ECM proteome. Current techniques show promise in that information of all expressed ECM proteins can provide a more complete map of the current physiological status of cells, tissues, organs, and whole organisms, which makes proteomics an ideal platform for identification of therapeutic targets [4]. Multiple approaches have been developed to enrich for ECM proteins, and we continue to develop methods that accelerate translation to the clinic. A context-specific biomarker, such as an ECM protein like collagen or fibronectin, is more likely to be feasible for translation than a biomarker identified by random sampling methods, and the cardiac ECM provides a rich source of such candidate biomarkers that have biological activity [4]. To fully realize the potential of proteomics, we need to bridge technological expertise required with an understanding of the most pertinent clinical questions. There is also a need to improve communication between the general research and proteomic research communities to help develop accurate expectations and perceptions of the processes involved.

Proteomics-based biomarker approaches can advance both prediction and management capabilities for cardiovascular disease. Streamlining proteomic techniques into the clinic can improve health status and effectiveness by directing strategies to those most likely to benefit while avoiding expensive or invasive treatments for individuals identified as being at low risk [4]. The use of proteomics in individual laboratories and in clinical research is a challenge, as cost of equipment, maintenance, and personnel involved in running the state-of-the-art MS instruments can be greater than the budget of a one grant lab [4]. Making MS more accessible and less technically challenging will allow biologists and clinicians to perform targeted proteomics and increase proteomic and biomarker research on a more global use scale. To achieve this goal, the proteomic field should borrow from the template of genomics who have made genomic capabilities, such as gene arrays, routine in most laboratories. Technological advancements such as miniaturization of equipment and simplification of hardware and software in addition to development of data analysis programs for proteomics datasets will diversify proteomic use. Recent advancements in techniques for ECM enrichment and enhancement of communication across disciplines will help to manage implementation and reduce the time required for large-scale projects.

A future direction for proteomics will be the development of methods that harness the large amount of big data need to acquire to determine mechanisms of action during development of cardiovascular disease. Proteins do not function as single units but as a dynamic network that communicate to influence pathophysiological state. Systems biology is a computational technique that focuses on characterizing the underlying network structure and dynamics of protein interactions. Qualitative systems biology focuses on network topology, characterizing the links and nodes of a communication network. This includes paths from inputs to outputs, the total number of paths, reachability, redundancy, and cross talk.

Quantitative systems biology measures and models kinetic parameters of a communication network. This analysis has been used to build a framework for macrophage activation and rate of infiltration stimulated by TGF β 1 in a mouse post-MI [83]. Due to the sheer depth, complexity, and size of datasets retrieved from proteomics, systems biology is the future for analyzing proteomic datasets and identifying possible therapeutic target.

5. Five-year perspective on advancements in ECM proteomics

There are several directions in which ECM proteomics are headed. For one, as more ECM proteins are identified and cataloged, targeted proteomics strategies can be developed for measuring multiple proteins using MS-dependent and MS-independent technologies. This approach will make proteomics more accessible and allow clinical investigations to advance on a more global big data scale.

A common feature of ECM that is highly desirable is easy accessibility of the target for drug binding, making ECM proteins highly amenable to intervention [84,85]. The complex network of collagens, elastic fibers, proteoglycans, and adhesive glycoproteins are aggregated, cross linked, and glycosylated, which need to be considered during drug development [2,4,14,17]. For this reason, understanding the spatial orientation and availability of target proteins for selection of optimal antigen sites is moving to the forefront in drug discovery. Whether aging influences drug accessibility to ECM targets needs to be investigated more, as aging is known to increase ECM cross-linking and decrease ECM solubility [86–91].

Understanding how ECM is regulated and how it regulates cellular processes, including inflammation and apoptosis, is needed. Proteomics has provided a useful means to understand MMP roles during cardiac aging in post-MI wound healing, including the large number of inflammatory mediators processed by MMPs [17,32,49,87,90,92–94]. Mapping protein–protein interaction networks (the interactome) will be important to fully explain biological systems. For example, in apoptosis, caspase cleavage was thought to initiate protein complex disassembly and cell death, yet ECM changes are strong inducers of the apoptotic pathway. Proteome-wide evaluation of proteolytic processing using the terminal amine isotopic labeling of substrates (TAILS) technique revealed little correlation between proteolytic and interactome changes, indicating that significant interactome alterations occur before and independently of caspase activity. Understanding the connections between the intracellular and extracellular environments will provide information on molecular and cellular physiological processes governed by protease–inhibitor interactions. Using computational approaches will provide insight into the complexity of protease–substrate interactions [95].

The development of noninvasive tests that accurately and reliably predict the development of heart failure before signs and symptoms occur will help improve patient outcomes. Of particular importance are the signaling cascades initiated by MMP generated ECM peptide fragments. MMPs generate fragments from substrates proteolysis, and these fragments are involved in a number of biological processes, including regulation of inflammation, angiogenesis, and fibrotic scar formation [17,32,96,97]. MMP cleavage products may serve

as novel and more selective treatment strategies for post-MI patients; more translational research is needed to understand how downstream products of MMP proteolysis alter physiological status [8,49,98]. As more MS data are acquired, big data approaches will be useful for harnessing these results to map biological networks [99–103].

In conclusion, using proteomics technologies to target ECM proteins has dramatically improved over the last decade allowing researchers to move away from observational proteomics and toward physiological proteomics. This will help in identifying novel targets and understanding the mechanisms of post-MI remodeling. Integrating proteomic technology into the clinic as a standard therapeutic approach will enable the field to move toward personalized medicine. The roadmap for clinical proteomics has been developed and can be successfully applied to a focus on cardiac ECM.

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Key issues

- Coupling ECM proteomics to physiological outcomes is the current gold standard for evaluating cardiac remodeling.
- Enhanced sample preparation protocols, as well as increased capabilities in mass spectrometry (MS), database searching, and bioinformatics analysis renders evaluation of cardiac ECM highly available for assessment.
- Integrating proteomics technology into the clinic as a standard therapeutic approach will enable the field to move towards personalized medicine.
- Understanding spatial orientation and target protein availability is necessary for selection of the optimal drug site during development of pharmaceuticals.
- MMP substrate cleavage products may serve as novel selective treatment strategies for post-MI patients; more research focused on downstream products of MMPs is needed.

Table 1

Evolution of extracellular matrix (ECM) proteomics.

Strategy	Pros	Cons
Strategy 1: Natural enrichment strategy [20,21]	Can identify more abundant ECM proteins in the infarct	Still has large amount of intracellular proteins
Strategy 2: Decellularization/differential solubility-based approach [3,22–24,97]	Provides unbiased first pass to focus on ECM changes that are cause and consequence of LV remodeling	Large sample to sample variation due to multiple extraction steps
Strategy 3: Sample fractionation [43]	Removes highly abundant proteins like albumin	Also removes some low abundant cytokines, lipoproteins, and peptide hormones that may be bound to albumin
Strategy 4: Hydrazide bead glycoprotein enrichment [31,32,35]	Eliminates highly abundant, non-glycosylated proteins	Differences refers to amounts of glycosylated protein and may not reflect total protein amounts

Over the past 20 years, we and others have developed and refined ways to enrich for ECM.