

Developments in Cardiovascular Proteomics

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Abstract

Cardiovascular diseases (CVD) are the leading cause of mortality and morbidity worldwide. These diseases can occur rapidly as seen in acute myocardial infarction or develop over a number of years as seen in chronic heart failure. The use of a number of drugs for chronic conditions in our aging population is compounding existing rates of CVDs due to cardiotoxicity. Recent advances in proteomics coupled with innovative separation and isolation techniques of subproteomes have vastly improved our knowledge of the cardiac proteome. Mass spectrometry imaging (MSI) is further providing information on spatial distribution of proteins and drugs. Here, we evaluate current advances in separation and detection of proteins, drugs and active metabolites being used to gain insights into mechanisms and diagnostic markers. Taken together these innovative techniques provide the necessary tools to investigate a system biology approach into cardiac disease and toxicity.

Keywords: Cardiac; Disease; Cardiotoxicity; Sub-proteome; Mass spectrometry imaging

Introduction

Cardiovascular disease (CVD) is the leading cause of global morbidity and mortality, causing approximately 30% of all deaths in the United States [1] and the UK [2]. The on and off-target cardiovascular system (CVS) effects of a multitude of drugs for different of conditions; including beta blocking agents, chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) [3-5] and anti-cancer agents add to the burden [6,7]. As the treatments for acute complications of CVD continue to improve, the epidemiology of CVD in our aging population is rapidly evolving from acute conditions to chronic disease.

An in-depth understanding of both the healthy and distressed CVS will allow greater understanding of the processes and mechanisms that may go awry in CVD. Many recent studies have used transcriptome and RNA profiling to describe CVD processes [8-10] and search for biomarkers of disease [11,12]. While these studies have shown vital aspects of molecular changes in CVD it is essential to understand the impact disease has at a protein level. Studies have shown in humans that not only do differences exist between DNA and the final mRNA product [13,14], but there is also marked variation in the expected proteome [15]. Consequently, a thorough understanding of the cardiovascular proteome is crucial to elucidating disease progression, biomarkers and potential therapeutic targets of CVD. Complicating things further, numerous studies point at post-translational modifications that not only regulate key processes in the CVS [16-18] but are also responsible for CVD pathologies and progression [19]. Recent innovative techniques using novel proteomic technologies to enrich and separate sub-proteomes have advanced our knowledge of disease progression and enhanced our ability to identify markers of disease and targets for new therapeutic strategies.

Gel based proteomics

Early examination of cardiac proteins were extensively carried out using two-dimensional gel electrophoresis (2DE) techniques and although these techniques have been predominantly replaced by gel-free methods, key studies still employ this well-established system [20]. In this technique, homogenized samples of cardiac tissue are solubilized and denatured, then separated based on isoelectric point and molecular mass using sodium dodecyl sulphate-polyacrylamide

gel electrophoresis (SDS-PAGE) [21]. In this technique, which offers a method to reduce the complexity of the entire sample, protein bands or spots are excised out of the gel and further analyzed by tandem mass spectrometry. This approach has led to fundamental advances in healthy cardiac proteomics [22,23], stem cell derived cardiac maturation [24], in-depth organellar proteomes [25,26] as well as changes that occur upon disease [23,27-30] and those that are induced by drugs [31]. In particular this technique provides a simple method of detecting post-translational modifications such as acetylation, glycosylation, phosphorylation and deamidation, in which there has been a shift in molecular weight and isoelectric points of the protein [20]. Methods utilizing 2DE do however tend to be biased against proteins with hydrophobic regions such as membrane proteins and biased towards high abundance proteins. 2DE also has a limited dynamic range of 10^4 magnitude [32,33] compared with the very high dynamic range of protein abundance, estimated at 10^6 for cells and tissues [32] and 10^{12} for plasma [23,32,34]. In an effort to overcome these biases, new separation methodologies as well as advancements in mass spectrometry instrument technologies have reduced the need for separating proteins using a gel based system (Figure 1).

In-depth analysis of the cardiac sub-proteomes

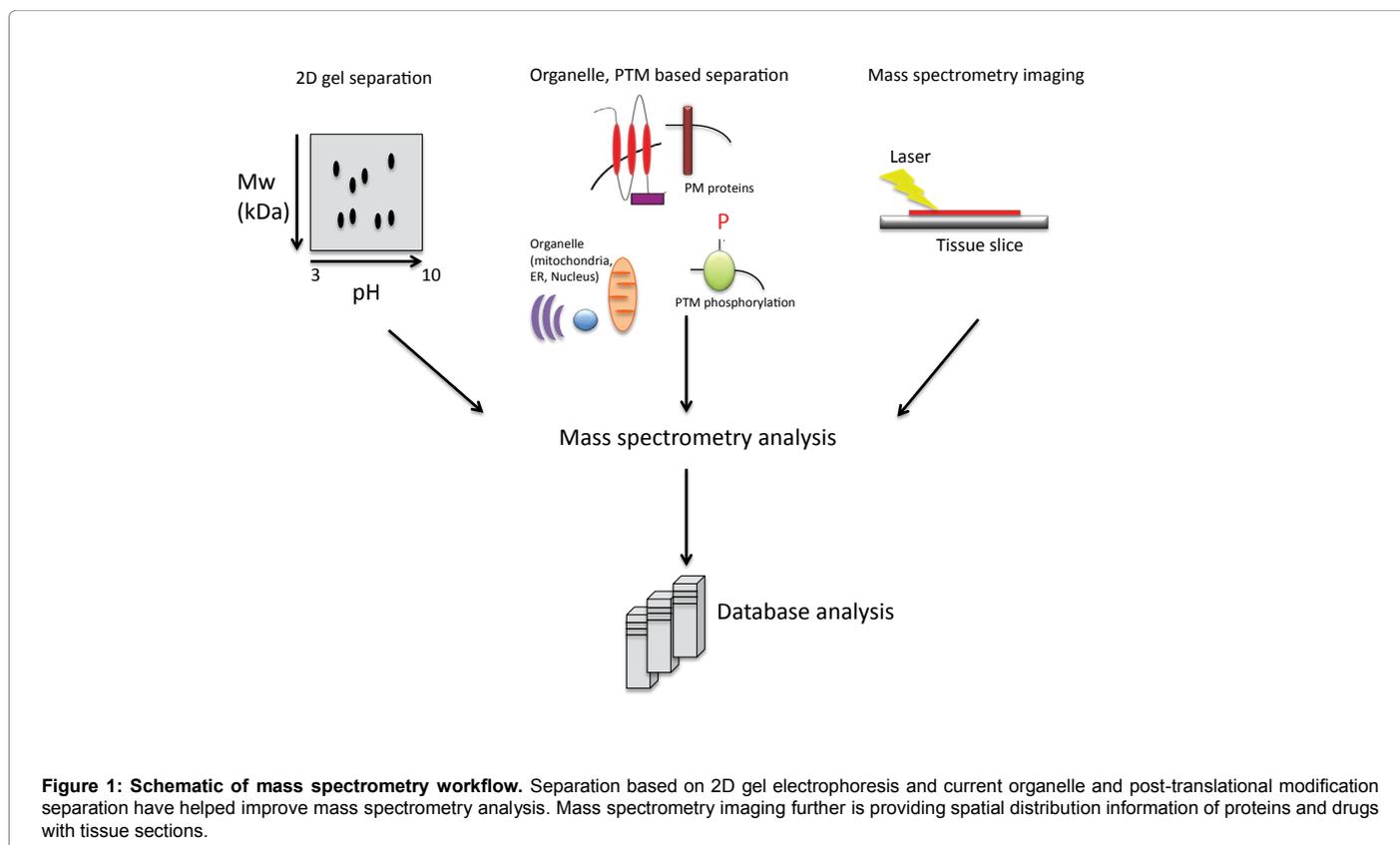
In an effort to analyze the cardiac sub-proteomes and in-turn reduce the complexities of the samples, several subcellular fractionation methods exist which first fractionate the samples. These methods include differential centrifugation, flow cytometry, immune-based isolation, membrane protein enrichment strategies and/or density gradient isolation of organelles. These techniques have been applied to study the sub-proteomes of cardiac sarcomeric enriched fractions [35], myofilaments [36,37], mitochondria [38-41] and nuclei [41,42].

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One key sub-proteome however that often gets overlooked is cell-surface proteins. These proteins are difficult to isolate and enrich due to their low abundance and relatively low solubility in aqueous media. This challenge has prompted us and others to develop and modify cell surface enrichment methodologies that can be coupled to mass spectrometry techniques to identify existing and novel proteins in this key cellular compartment.

Cell surface proteomics

The cardiomyocyte plasma membrane is essential to the function of the heart, from the spread of an action potential to the regulation of inotropic and chronotropic changes of the heart. Analyzing cardiac membrane proteins is crucial to a better understanding of heart function especially when we consider that the majority of cardiac disorders including arrhythmias, cardiomyopathies and conduction defects can be attributed to altered expression, function, or subcellular localisation of cardiac ion channels and associated membrane proteins [43-46]. Thus the identification and characterization of membrane proteins is paramount to the understanding of cardiac dysfunction and the search for therapeutic targets. With this in mind, several techniques have been developed recently to isolate and purify plasma membrane proteins including: colloidal bead isolation, biotinylation and glyco-capture, each having its own advantages and limitations which will be discussed in turn.

Colloidal silica bead membrane isolation: The silica bead plasma membrane isolation procedure uses positively charged colloidal silica beads to coat and bind to the anionic plasma membrane, and following homogenization and centrifugation, the plasma membrane is stripped from the rest of the cell [47] and then further purified in a discontinuous

nycodenz gradient. Recently we refined this protocol in order to couple it with shot-gun proteomics [48] and used it to identify plasma membrane proteins *in vivo* [49] and *in vitro* [50]. Our *in vitro* analysis of the mouse and human cardiomyocytes identified a total of 3033 mouse and 2762 cardiac proteins. This study method identified a total of 555 membrane associated proteins which included Tmem65, a novel cardiac membrane protein shown to regulate essential cardiomyocyte functionality [50]. This isolation procedure is however limited by its tendency to rupture cells, especially cells in early developmental stages which may have a dynamic membrane due to morphological immaturity and cause them to leak. This allows the beads to enter the cell and bind to internal organelle membranes thereby significantly contaminating the plasma membrane fraction [47,51].

Biotinylation: Biotinylation is used to isolate cell surface proteins by exploiting the strong affinity of biotin for avidin. Preparations of cell-impermeable, modified biotin allow it to bind to primary amines of exposed proteins in a cell culture system or using the *in vivo* application those accessible via the blood stream [52,53]. The proteins can then be isolated with avidin beads and following elution from the biotin-avidin complex these proteins can be identified using mass spectrometry [53]. We have previously utilized this technique to identify cell surface markers on lineage specific stem cells, isolated from mouse embryos [54] which results in their complete separation using fluorescence assisted cell sorting (FACS). More recently, Strassberger et al. [55] used biotinylation combined with mass spectrometry to extract differentially expressed cell surface proteins in myeloid leukemia cell lines compared to normal human granulocytes. They identified a number of proteins found preferentially in myeloid leukemia cells and were able to target one of them, namely CD166/ALCAM, to kill these cells *in vitro*. This

methodology is limited however in its ability to extract cell membrane proteins without an exposed extracellular domain thereby biasing against membrane proteins that are associated with the intracellular face of the plasma membrane and proteins fully enveloped inside the phospholipid bilayer.

Glycosylation: Glycosylation is the most common post-translational modification [56] and aberrant glycosylation has been shown to be early indicator of cellular changes in a multitude of human diseases including cancer, inflammation and neurodegeneration [57-59]. Alterations in plasma membrane glycoproteins have also been reported in ischemia reperfusion injuries [60] and cardiac hypertrophy [61]. Most membrane-bound and secretory proteins synthesized by mammalian cells are glycosylated and thereby may provide useful diagnostic tools as proteins that are shed from the cell surface during disease as well as offer mechanistic insights into disease progression and pathology. This understanding has thereby stemmed a wide variety of techniques to specifically isolate cell surface and plasma secreted glycoproteins. These include separation based on hydrophilic interactions [60,62,63], exploiting the high affinity of glycosylated proteins and peptides for lectins and the chemical capture of glycoproteins using hydrazide technology. A variety of lectins are available that selectively bind to different oligosaccharide epitopes and thereby can specifically isolate for a unique sub-set of glycoproteins containing a specific glycan moiety [59]. Although no one lectin is capable of isolating a cell's complete glycoprotein complement, studies are now combining multiple lectins to achieve greater coverage [59].

Hydrazide technology aims to isolate a broader range of glycoproteins. This procedure involves oxidizing carbohydrates, to convert cis-diol groups into aldehydes so that they may be linked to hydrazide groups on solid supports, leading to their enrichment [56]. This technique was further refined for use in live cells by Wollscheid et al. [64] who showed that the gentle biocytin hydrazide labelling of oxidized carbohydrates is cell impermeable and can therefore be used to characterize changes in the cell surface glycoproteome. This procedure, termed cell surface capture (CSC), has recently been used to establish a cell surface protein atlas (CSPA) in which researchers have used CSC technology to isolate surface proteins from 41 human and 31 mouse cell types providing an invaluable cell surface resource [65]. A study conducted by Parker et al. [60] used multiple methods to enrich for glycoproteins in myocardial ischemia and reperfusion injury and identified extracellular and cell surface proteins involved in the various stages of cardiac remodeling. Their study suggested that cardiac remodeling following myocardial ischemia may begin much earlier than originally hypothesized. In a more recent study Yang et al. [66] identified 32 glycosite containing peptides elevated in a canine model of dyssynchronous heart failure of which 13 glycopeptides were reverted to normal levels after cardiac resynchronization therapy [66]. The major drawback of hydrazide based techniques is that efficient detection of glycosylated proteins requires the removal of the oligosaccharide moieties from the glycoprotein [67]. For N-glycosylation the β -aspartylglycosylamine linkage can be selectively cleaved using peptide-N-glycosidase F (PNGaseF) but until recent developments those moieties in which the root β -1,4-acetylglucosamine had been fucosylated were missed in the analysis [68]. Recent protocols however, have used *Lens culinaris* Agglutinin (LCA) to enrich for core fucosylated (CF) proteins, followed by Endo F3 partial deglycosylation and NanoLC-MS/MS. This identified CF proteins in human serum from healthy volunteers and patients with pancreatic cancer [69] and hepatocarcinoma [70]. This technique provides an essential tool to ensure greater coverage of the glycoproteome and indeed Parker

et al. [60] demonstrated that a combination of techniques to enrich for glycoproteins offers greater depth. O-linked glycosylation further lacks a common moiety, leaving its cleavage more challenging and to date a cocktail of glycosidases have been used [68]. A lack of consensus currently limits their detection and thus the majority of studies have focused on N-linked glycoprotein analysis [59].

Phosphoproteomics

When discussing cardiac sub-proteomes it is hard to ignore the phosphoproteome. Reversible protein phosphorylation is not only vital for the normal coordinated function of the heart but is also implicated in disease initiation and progression. This post-translational modification is essential for propagating cell signaling events in response to hormones, second messengers and pharmacological agents.

Several methods exist to subtract phosphorylated proteins from the cellular milieu which include both in-gel and gel-free methods. Using 2D-gel electrophoresis a visual migration of the protein can be monitored due to additional charge of the phosphorylated group which can then be specifically excised from the gel, purified and then identified by mass spectrometry methods. A recent study using 2D-gel electrophoresis identified 22 proteins with significantly changed phosphorylation states expressed during the acute off-target cardiotoxic effects of the chemotherapeutic agent doxorubicin. This study highlighted key signaling pathways involved in energy metabolism, sarcomeric function/structure and chaperone activity which could lead to identification of the early events involved in cardiotoxicity [71].

As previously mentioned, there has been a deviation from gel based methods and gel-free methods are predominantly used for phosphoprotein and peptide enrichment which include the use of titanium dioxide beads and immobilized metal affinity chromatography (IMAC) combined with mass spectrometry [72]. Titanium dioxide has a very high affinity for phosphopeptides and has been shown to be able to isolate femtomoles of phosphopeptides from complex protein mixtures [73,74]. Prior to the identification of proteins and signaling pathways involved in disease processes, it is essential to assess the healthy state. Lundby et al. [75] used titanium dioxide phospho-enrichment coupled to LC-MS/MS, to quantify the changes in the mouse cardiomyocyte phosphoproteome *in vivo* upon acute beta1-adrenergic and beta2-adrenergic stimulation. They uncovered 670 phosphorylation sites regulated by beta1-adrenergic stimulation which included previously unknown modifications of channels and transporters affecting cardiac function, highlighting the utility of phosphoproteomics analysis for elucidating cardiac signaling networks [75,76]. Wijeratne et al. [77] describe a novel workflow involving reductive alkylation by acetone to label peptides in tandem with titanium dioxide isolation followed by mass spectrometry based identification to effectively quantify phosphorylative changes in a mouse model of ischemia-reperfusion injury. Using this methodology they found increased cardioprotection in a mouse model of ischemia-reperfusion injury was due to low molecular weight fibroblast growth factor-2 (LMW FGF2) mediated phosphorylation of key downstream signaling pathways [77].

Quantification

Although an in-depth review of protein and peptide quantification is outside the scope of this review they have been reviewed extensively elsewhere [23,78-80]. The most commonly used techniques for peptide quantification used in mass spectrometry can be divided into those that offer a relative quantification between healthy and diseased/treated samples and those that offer absolute quantification. Label

free relative quantification techniques include spectral counting, peak intensity comparisons and more recently SWATH analysis [50,79,81-83] whereas labelled techniques include SILAC, iTRAQ, enzymatic ^{18}O labelling and *in vivo* $^2\text{H}_2\text{O}$ labelling [79,84-87]. Absolute techniques incorporate the use of internally spiked absolute quantification (AQUA) peptides to quantify absolute amounts using MRM, SRM, iTRAQ [88-91]. These techniques have been extensively used in cardiovascular proteomics [23,78,80] and can be combined with a number of the sub-proteome separation techniques in which quantification of differentially expressed proteins are informative about mechanism of disease progression, pathway analysis of potential drug targets and biomarkers. For example, the quantitative accuracy of SWATH analysis has been compared to that of SRM analysis [92] and has the ability to reproducibly identify low abundance proteins from small numbers of cells (50 000 cells) and from small amounts of tissue from biopsy samples (0.2-0.5 mg) including cardiac tissue [92]. SWATH and SRM were used in combination with CSC glycoprotein enrichment methods to provide quantitative and qualitative expression of 78 human and mouse tissue cell surface proteins [65]. Lau et al. combined *in vivo* $^2\text{H}_2\text{O}$ labelling of drinking water with subcellular fractionation to quantitate protein dynamics and turnover in healthy mice and those with cardiac hypertrophy. This invaluable resource of cardiac disease progression provides quantification of the *in vivo* half-lives of 3,228 proteins and the expression of 8,064 proteins under healthy and diseased states [86]. Lam et al. [93] combined differential centrifugation to isolate mitochondria from human and mouse hearts with TiO_2 purification of phosphoproteins with MRM quantification to establish 176 MRM transitions specific to protein modifications. These transitions can now be used to quantify alterations in the level of phosphorylation under conditions of disease or pharmacological intervention.

New mass spectrometry technologies

The advancement of mass spectrometry instrumentation and computational technologies has vastly improved the coverage and depth of the proteome. A comparison of generations of the Q Exactive instrument for example, showed that the latest iteration of the Q Exactive HF has the capability to detect > 4000 proteins in a 1 hour gradient from 1 μg of Hela cells prior to any fractionation [94]. These powerful methods offer great versatility and depth when dealing with precious samples, in particular healthy human tissues which can not only be rare and difficult to obtain but also only available in small quantities retrieved from patient biopsies. These restrictions therefore limit the amount of sample manipulation and fractionation prior to analysis. Indeed, we have used these powerful methods to identify 2754 human atrial protein groups and 2825 human ventricular protein groups from which we were then able to identify and classify chamber unique proteins without prior fractionation [95]. If however the initial sample quantity does lend itself to prior fractionation, the possibilities are extensive as demonstrated by TiO_2 enrichment of phosphoproteins which when analyzed gave 7600 unique phosphopeptides in a 1 hour gradient of elution [94].

Mass spectrometry imaging: One limitation of sub-fractionation is the loss of spatial distribution of proteins and drugs. Emerging technologies of mass spectrometry imaging (MSI) are now providing a powerful tool that allows the analysis, detection and mapping of the biodistribution of proteins and drugs within histological sections [96]. MSI can detect endogenous and exogenous analytes including low molecular weight drugs, lipids and proteins directly from tissue samples [96] (Figure 1). This technique has traditionally been linked to MALDI ionization and accomplished by using tissue sections

mounted onto a conductive slide in water or gelatin to which one of several MALDI matrices are sprayed depending on the type of analyte to be measured [96]. The matrix then co-crystalizes with the cell components and then the region of interest is subjected to MALDI ionization for detection by mass spectrometry [96,97]. More recently MSI has evolved to use desorption electrospray ionization (DESI) [98], which overcomes complications of matrix-analyte co-crystallisation issues [99]. The technical aspects of MSI and sample preparation are beyond the scope of this review but have previously been discussed in depth [96-99]. Using these techniques Martin-Loenzo et al. [100] pinpointed specific lipids and proteins to atherosclerotic aortic mouse cardiac sections. They showed that thymosin beta 4 is differentially overexpressed in the intima layer of atherosclerotic tissue compared to media layers providing new avenues of research in the area [100]. This technique further holds a lot of promise in drug distribution analysis.

Histological sections of models of drug dosed tissue allow the detection of drug and metabolite distribution. MSI has recently been used to monitor and quantify the release of celecoxib from packaging nanoparticles delivered to the healthy and ischemic myocardium of *in vivo* models. This study identified the angiogenic potential of this drug when delivered locally to a specific region, pointing to a potential new approach for treating ischemia [101]. Drug-induced cardiotoxicity remains the major contributor to drug attrition and withdrawal from patient distribution and a major complication for off-target toxicities of a multitude of drugs [6]. Whole body MSI offers the promise to determine localization of both parent and metabolite distribution within every organ or cell type of the body early in drug development stages, potentially aiding drug testing and reducing adverse off-target reactions [96,102,103]. One major drawback of MSI is the inability to directly identify proteins and the potential need to coordinate experiments with tandem mass spectrometry methods to carry out sequence identification [104,105]. However, with the continuing development of technologies, databases are now being established by the MSI community that allows the identification of molecules directly from the mass spectrometric measurement [106]. The identification of proteins [107,108] and glycoproteins [109,110] as well as lipids and cell metabolites [106] from MSI experiments of healthy versus diseased or pharmacologically treated tissue will undoubtedly reveal deeper insights into disease mechanisms and pathways at a tissue level.

Conclusions

This review highlights current technologies that have been developed and modified to further cardiovascular knowledge through proteomics. Taken together, these studies highlight a systems biology approach allowing the identification and quantification of potential targets, biomarkers, and mechanisms of disease progression including identification of post-translational modifications as well as methods to gain insights on the biodistribution of parent drugs and active metabolites. Applying the correct innovative techniques for a given system under healthy, diseased and drug-dosed conditions will allow us to gain further understanding into mechanisms that cause disease and drug-induced cardiotoxicity. These insights will in-turn help us to gain a deeper comprehension into cardiac disease and to reduce the number of drugs that can cause adverse cardiac reactions before they enter patient circulation.

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