Proteomics

High-Resolution Separation of Cardiovascular Proteomes
Two-Dimensional Electrophoresis and Liquid Chromatography

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With 2-dimensional electrophoresis and liquid chromatography (LC)-based proteomics firmly established, scientists are provided highly advanced and reliable tools to take major steps toward understanding functional networks leading to cardiovascular disease. This review aims to provide an educational background for 2-dimensional electrophoresis and liquid chromatography-based separation, enabling cardiovascular researchers, in clinical as well as in basic research, to obtain results with high quality regardless of their current experience in the analysis of proteomes. The review begins with an introduction to high-resolution 2-dimensional electrophoresis and liquid chromatography and their capabilities. The biological role and analysis of protein heterogeneity is discussed in detail because 2-dimensional electrophoresis is especially suitable for visualizing distinct forms of proteins at high throughput and is still the method of choice for quantitative comparison of intact proteins. It continues with an overview of what can be expected from in-depth proteome analyses in terms of proteome coverage, leading to a discussion on the major challenges in proteomics, such as the tremendous differences in the abundance of cardiovascular proteins. The latter includes suggestions for tackling difficulties encountered even by experts, for example, during detection. A particular focus is provided on the current trend of subproteome analyses, which encompasses studies from whole organelles down to single protein complexes. Often underrepresented, a brief introduction to software analysis and content management is given. The review concludes with a future perspective on nondenaturing proteomics, which will provide most accurate insights reflecting the physiological role of proteins, as first steps in this direction have demonstrated. In summary, the review explains concepts and discusses approaches, which are fundamental for gaining highly competitive results via high-resolution separation of cardiovascular proteomes.

Although DNA and RNA provide inherited templates for life, proteins are the effectors, defining structure and function from the molecular level to all that is visible and physically examinable in a patient. Prominent examples pointing out the versatility of protein expression refer to the fact that all cells of an individual have basically the same genome, but distinct sets of functional proteins. All proteins expressed in a cell type or organ at a defined time of analysis are referred to as the proteome (eg, the cardiac proteome of healthy adults). Complete proteomes are thought to contain thousands to several tens of thousands of distinct proteins.

In contrast to analyses of genomes, no complete proteome has been published yet. The depth of a proteome analysis is considered one of the greatest challenges in proteomics, and the number of analyzable proteins is highly dependent on the quality of protein separation. All current approaches for detection require either spatial (gel electrophoresis) or temporal (chromatography) separation. Thus, the adequacy of protein separation determines the completeness of proteome analyses. Two-dimensional electrophoresis (2-DE) and liquid chromatography (LC) are particularly suitable for high-resolution separation in proteomics and enable the detection of several thousand proteins in a single experiment.3-5

Seperating Cardiovascular Proteomes at Highest Resolution by 2-DE and LC

2-Dimensional Electrophoresis

A comprehensive approach to cope with the tremendous number of proteins in a cardiovascular sample is to achieve protein, or in case of online LC, peptide separation at highest resolution. By combining widely-used SDS-PAGE subsequent to isoelectric focusing, 2-DE makes use of 2 individual technologies to separate thousands of proteins on a single gel.6-8 In SDS-PAGE, the resolution is generally selected according to the molecular weight of proteins by adjusting the acrylamide concentration. However, specialized protocols require modification of additional parameters, such as crosslinker to acrylamide ratio. Similarly, parameters are adjustable for isoelectric focusing and by combining them can improve the resolution by several folds. For example, using overlapping narrow immobilized pH gradients for 2-DE can improve the number of resolved protein spots by up to 3-fold.3,5 Thus, adjusting parameters for isoelectric focusing is considered more essential in terms of resolution than for SDS-PAGE. An excellent overview of such parameters and good introduction to 2-DE was published by Gorg et al.1,6,9

As with any technology, optimization of parameters is essential for 2-DE, but once established, high-quality data can be obtained even for proteins with extreme biochemical properties, such as alkaline or membrane proteins.1,10-12 Thus, it has been demonstrated that 2-DE electrophoresis is suitable...
to separate cardiac proteins at high resolution with isoelectric points from 3 to 11 and molecular weights from 10 to 100 kDa in routine analysis.\textsuperscript{5,7,8,13} A recent study, analyzing >2000 protein spots by 2-DE in a context of atrial fibrillation, identified alterations in protein abundance as well as post-translational modification (PTM) of structural and heat shock proteins.\textsuperscript{14}

**Liquid Chromatography**

Analog to 2-DE, LC is dependent on numerous parameters that critically influence resolution and separation. In proteomics, LC is predominantly coupled online with mass spectrometry (MS) using C18 columns (stationary phase) for hydrophobic interactions and an increasing concentration gradient of organic solvent (eg, acetonitrile; mobile phase) for elution (reverse phase LC). Flow rates, column dimensions, and surface area are among the critical parameters for highest resolution.\textsuperscript{15,16} Technology has evolved toward the end of nanoscale flow rates to minimize the required sample amount while further improving separation, and is nowadays found in most routine MS laboratories.\textsuperscript{16,17}

Besides reverse phase LC, several other LC approaches have been used to analyze proteomes successfully. Similar to 2-DE, 2 LC separation technologies (strong cation exchange and reverse phase) have been combined in an approach named multidimensional protein identification technology (MudPIT) to achieve highest resolution in an effort to map proteomes more completely.\textsuperscript{4} Applied to the heart, MudPIT supported the identification of nearly 5000 proteins.\textsuperscript{18}

Although predominantly used for peptide separation after tryptic digest of a sample, LC is well capable of separating intact proteins or even protein complexes (Table). However, the suitability for online MS of intact proteins in a proteome-wide analysis is rather poor, because it does not yet have the capability to analyze hundreds or thousands of proteins without intermediate purification (eg, de-salting) or concentration. Therefore, the sample requires fractionation during elution and subsequent processing. In this manner, >150 human serum proteins were identified, including distinct troponin T isoforms.\textsuperscript{15} The novelty of this approach was the use of chromatofocusing, which also separates proteins according to their isoelectric point. At that stage of the multicentered human plasma proteome analysis, the study added 81 proteins to the list of previously identified proteins.\textsuperscript{20} The effort to map the human plasma proteome pointed out major limitations regarding the lab-to-lab comparability of proteome analyses, as roughly 20% of the proteins were identified by at least 1 source and only about 5% by all 4 compared sources.\textsuperscript{20} Neglecting the intrinsic false discovery rate of MS analyses, this unexpected result emphasizes how critical sample preparation, resolution, and instrumentation influence the results in proteomics and substantiates the use of complementary approaches.

Besides downstream application of LC-MS after 2-DE, it has been successfully used after SDS-PAGE of intact cardiovascular proteins, eg, to describe cardiac proteomes of drosophila and the porcine extracellular matrix.\textsuperscript{21,22} Typically, a lane is subdivided into equal rectangles from top to bottom, and digested by trypsin similar to that done for 2-DE gel spots. However, precious information that could be gained from such processing, like the approximate molecular weight, is rarely incorporated in proteome analyses and content management (see section titled Software Analysis).

The reviewed examples demonstrate that 2-DE and LC both support the analysis of thousands of proteins. However, LC-based proteomics approaches are mostly based on prior digestion of the sample into peptides. Because separation by 2-DE and LC is based on distinct biochemical properties of proteins/peptides, the technologies are rather complementary in their effectiveness to separate cardiovascular proteomes. Thus, any comprehensive study would benefit from using both technologies to maximize the number of analyzed proteins, including their distinct forms, which is referred to as protein heterogeneity in the following paragraph.

### Analysis of Protein Heterogeneity

A critical point easily overlooked in discussions comparing 2-DE–based analyses with currently prevalent LC-MS–based analyses is the fact that the latter mostly analyze digested proteins. Thus, information about a protein being nascent, differently processed or posttranslationally modified, as well as already being partially degraded, will be generally lost. For example, it is indistinguishable whether 2 PTMs identified by LC-MS on a digested protein originate from 1 form of the protein or 2 separate forms, except that they are identified on the same peptide. Therefore, LC-MS–based analyses

| Table. Use of Major Separation Technologies for Proteomics |
|-----------------|------|--------|--------|
| Proteomics of | 2-DE | DIGE | Offline LC* | Online LC |
| Peptides | — | — | ++ | +++ |
| Intact proteins | +++ | +++ | ++ | + |
| Protein complexes | ++ | ++ | +++ | ++ |
| Protein identification† | ++ | ++ | +++ | ++ |
| Immunodetection | +++ | +++ | ++ | — |
| PTMs‡ | +++ | +++ | ++ | +++ |
| Chemical modification | ++ | ++ | ++ | +++ |
| Protein size/coverage | +++ | +++ | ++ | + |
| Comparative studies | ++ | +++ | + | ++ |
| Subproteomes | +++ | +++ | ++ | +++ |

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‡ Indicates 2-dimensional electrophoresis; DIGE, difference in-gel electrophoresis; LC, liquid chromatography; PTM, posttranslational modification; —, not suitable; +, suitable; ++, good; and +++, excellent publication record in the field.

*Offline LC refers to manual transfer to mass spectrometry analysis, which limits throughput and resulted in a penalty for most applications.

† Requires prior purification of protein complexes (eg, by LC) and generally involves denaturation before separation.

‡ All offline technologies received a penalty because of limited throughput regarding protein identification. Still, the compatibility with immunodetection or specialized chemistry (eg, oxyblot) for identification of proteins or PTMs in combination with the information about isoelectric point and molecular weight delivers hitherto unmatched information about proteomes.
will not reveal that individual proteins exist in >1 form in proteomes.

The prevalence of protein heterogeneity becomes apparent even by a casual inspection of any given 2-DE–based analysis. For the plasma proteome, fractionation by LC and subsequent 2-DE analysis demonstrated that 325 proteins were represented by a manifold number of protein spots on a 2-DE gel, demonstrating heterogeneity of numerous proteins. For the cardiac proteome, a seemingly extreme case of single protein heterogeneity was reported for myocardial Hsp27, which was represented by up to 59 individual protein spots on a 2-DE gel. Artificial heterogeneity, such as fragmentations, should be considered and addressed in preliminary experiments during optimization of sample preparation. Remarkably, a subsequent comparative study indicated that 12 Hsp27 spots were altered in dilated cardiomyopathy and 10 in ischemic heart failure. Hsp27 seems to confer protection against ischemia, which was linked to its phosphorylation. Another 2-DE–based study on cardiac ischemia supported the notion that alterations in PTMs modulate protein function as much as protein abundance, because the detection of distinct phosphorylated forms of mitochondrial aldehyde dehydrogenase 2 correlated with an increase in its enzyme activity and cardioprotection against ischemia via protein kinase Cε signaling. Altogether, there is strong evidence that different forms of the same protein, which can be currently visualized by 2-DE, require close attention in proteomics (Figure, Table).

Future improvement for accurate identification of protein forms on 2-DE gels is required because, although protein identification has become routine in proteomics, the difference between ≥2 protein forms remains mostly obscure. The main reasons for making the differentiation between 2 protein forms challenging are that (1) 100% sequence coverage is rarely achieved, (2) protein modifications are potentially unstable during LC-MS analysis, and (3) it is not feasible to consider all types of PTMs in current software analyses, because they range in hundreds. Therefore, comprehensive characterization of protein heterogeneity poses one of the greatest challenges in the endeavor to achieve complete coverage of cardiovascular proteomes.

**Current Coverage of Cardiovascular Proteomes**

The human proteome is under intense investigation and considered to be far more complex than the human genome. Since late 2011, UniProt replaces the previous effort of the International Protein Index to track and archive the progress of mapping the human proteome. On the basis of the 70,000 entries stored in UniProt as of July 2012 and an analysis using the JVirGel freeware, most of the suggested human proteome falls in theory within the pl and Mw range that is analyzable by 2-DE (pI 3–11, Mw 10-100kDa; see section on 2-Dimensional Electrophoresis). In contrast, the protein spots reported on 2-DE gels range within thousands at most. Similarly, proteins identified by online LC-MS range in thousands.

Focusing on the plasma proteome as an example being under most scrutiny, results indicate that global proteomics approaches might have peaked in proteome coverage. In 2005, the Human Proteome Organisation (HUPO) Plasma Proteome Project initiative reported about 3,000 proteins being identified in an impressive multicenter collaboration. This number seems so far unsurpassed and was revised to ~2000 proteins with higher confidence in 2011 using advanced instrumental and computational models, indicating the trend toward higher confidence rather than sheer numbers.

A major reason for the difference of at least 1 order of magnitude between the number of proteins in UniProt and number of identified proteins in a global proteome analysis is that the database archives all proteins ever reported and information about tissue or developmental expression is limited. However, cardiovascular proteomes at a certain developmental or disease stage are only represented by a currently still unknown fraction of all proteins in UniProt. A major undertaking toward gaining knowledge about protein expression patterns using antibodies is performed by building the Human Protein Atlas. All developed antibodies are commercially available and will be powerful tools if combined with 2-DE or affinity LC, for example, in the identification of heterogeneity of a protein (see Analysis of Protein Heterogeneity; Table).

Another major reason for proteomes visualized by 2-DE and LC approaches being incomplete is the vast dynamic range of naturally occurring proteins, which is still unmatched by current detection technologies and discussed in the following section. As a solution, the analysis of fractionated proteomes, also called subproteomes or functional proteomes, developed into multiple highly successful approaches within the past decade (see Cardiovascular Subproteomes).

**Dynamic Range of Cardiovascular Proteomes and Current Technology**

In a landmark review on the blood proteome, Anderson et al pointed out that blood plasma contains proteins at concentrations ranging from 5 pg (interleukin-6) to 50 mg (serum albumin) per mL. Thus, the concentration of serum albumin in human blood plasma is ≥10 orders of magnitude higher than the concentration of interleukin-6. Similarly, contractile and structural proteins are among the proteins with highest abundance in the myocardium with serum albumin being a
potential contaminant, hence potentially saturating a separation and overshadowing proteins at much lower abundance, such as regulatory proteins. Examples demonstrating the significance of highest sensitivity for the identification and evaluation of prognostic marker proteins for risk stratification of acute coronary syndrome and cardiac ischemia are cardiac troponin I and T. Quantification of proteins in the ng to pg per mL blood plasma range is typically determined in routine clinical chemistry laboratories by highly sensitive immunoassays (eg, ELISA). In proteomics, it is achieved by enrichment, fractionation, or depletion, which is discussed in Cardiovascular Subproteomes.

The linearity of the dynamic range as well as sensitivity in current proteome analytics is highly dependent on the mode of detection. Linearity of the dynamic range is largely dependent on the detector. For routine 2-DE, the upper limit of the linear dynamic range seems to be 5 orders of magnitude by using fluorescent staining or labeling of proteins and laser densitometry (eg, Typhoon series). Widely used are ruthenium-based staining (commercially available under the name Sypro Ruby) and Cy-dye labeling. Attention is required when published protocols or products carrying those names are selected as they have become available from distinct sources, but have different sensitivity or a specialized function (eg, minimal versus saturation Cy-dye labeling, original versus new formulations of ruthenium-based stainings).

Indicated as nontoxic and biodegradable, a fluorescent dye named epicocconone is available (commercially: Deep Purple), which seems to stain proteins with similar sensitivity and dynamic range as ruthenium-based detection. Again, following a robust protocol is essential to achieve high sensitivity, because protein binding of epicocconone is highly pH dependent and seems to be maximally stable at pH 2. Therefore, a robust protocol should maintain an acidic pH after protein staining. Staining of the proteins itself is performed for 1 to 2 hours at alkaline pH, eg, in sodium borate.

Proteins separated by LC are generally detected by UV absorption, which ranges from 3 to 5 orders of magnitude. In particular, reverse phase LC in combination with nanoscale flow rates is widely used online with MS to achieve highest sensitivity in the nanogram range. Then, the dynamic range is limited by mass spectrometers as they are used as online detectors. Online operation of LC-MS systems requires instruments with fast acquisition preferably at high mass accuracy. The dynamic range for MS instruments with such capacity was indicated as high as 5×10^3. However, this range refers to the intraspectrum intensity, which limits the detection of peptides present at larger ratios when being eluted and enter the mass analyzer at the same time. In different spectra, the sensitivity of the mass spectrometer represents the lower limit of the dynamic range. The upper limit can be regarded as the saturation of the column used for LC. In case of saturation, high abundant proteins, such as albumin, continue to be eluted continuously during a separation and potentially contaminate even separations of subsequent samples on the same column.

As current proteomics technologies are still no match for the tremendous dynamic range of protein abundances in biological samples, multiple strategies have been successfully developed to navigate around those limitations. They all share the reductionistic approach of analyzing subproteomes (eg, of organelles or protein complexes), which has the potential to learn much more about a defined protein network than an unorganized protein mixture of whole cells or organs.

**Cardiovascular Subproteomes**

The challenge in proteomics to visualize proteins, which naturally occur at largely different concentrations in biological samples, is currently overcome by focusing on subsets of proteomes, such as mitochondrial or single protein complex subproteome, instead of full proteomes. This is facilitated by various purification approaches before proteome analyses. Most approaches follow 1 or a combination of 3 basic principles: (1) depletion of high abundant proteins, (2) enrichment of functional proteomes, and (3) combining multiple steps/dimensions for separation. In all cases, subproteomes with reduced complexity are being analyzed.

**Depletion**

Depletion of high abundant proteins was found most suitable for human plasma, because of the unusually high concentration of a few proteins, such as albumin, in a biological sample. Depletion is widely achieved by specific antibodies for high abundant proteins.

However, albumin is a well-known carrier protein and as such it was not completely unanticipated when multiple intact proteins were found to be associated with albumin after depletion. In principle, all proteins interact with others under in vivo conditions. This principle is successfully used in co-immunoprecipitation, which, in combination with 2-DE, is a powerful tool to identify associated proteins as exemplified for myocardial protein kinase Cε (Table). Thus, any depletion holds the risk of removing unknown proteins and requires careful consideration in a comparative study, in particular, when abundant proteins are suspected to change quantitatively.

**Enrichment/Purification**

Organelle, functional proteomes or complexomes all refer to the analysis of enriched subproteomes of a biological sample. Therefore, enrichment of subproteomes is a highly diverse field of research and would require multiple reviews to be comprehensive. Approaches for enrichment are highly versatile ranging from purification of protein complexes (including co-immunoprecipitation) to enrichment according to functional groups (eg, phosphorylation) or subcellular localization (eg, mitochondria; Table). As these proteins are consequently associated with a more or less specific function that is linked to the enriched fraction, the term functional proteomics evolved within this area of research.

Both 2-DE and LC have a special standing in the analysis of subproteomes, because LC is widely used for enrichment, whereas 2-DE enables the visualization of proteins with specific functional groups at high resolution even without prior enrichment. For example, oxidation of proteasome subunits was linked to proteasome dysfunction in human heart failure by a combination of derivatization of carbonylated proteins (oxyblot), 2-DE and western blotting (Table).

A major research focus is the mitochondrial subproteome. Several in-depth studies on myocardial proteins of the myocardium in comparison with those of other tissues or in the context of pathophysiological conditions were performed to derive novel functional information. Using 2-DE,
>1000 mitochondrial protein spots can be visualized and either quantitatively compared or probed for PTMs. Applied to the study of heart failure, 2-DE has been demonstrated to successfully identify proteins associated with improved cardiac function after resynchronization therapy. In a study on protein kinase C ε-mediated cardioprotection, a 2-D difference in-gel electrophoresis (DIGE) approach revealed altered abundance of proteins, which are responsible for removal of reactive oxygen species and the cellular energy supply, including membrane proteins. DIGE relies on pre-electrophoretic labeling of proteins with fluorescence dyes and is particularly useful for comparative analysis, because ≤3 samples can be separated on a single gel (Table). Therefore, it improves and simplifies the software analysis of 2-DE gels (see section Software Analysis).

Combining Subproteomes
The third and last principle of subproteome analysis refers to a combination of multiple parallel or consecutive steps to reduce the complexity of a biological sample. The major difference to the previously 2 reviewed principles is that several subproteomes are analyzed in parallel to combine them to a global proteome. This can be achieved via LC in a MudPIT-like offline approach or via 2-DE by using multiple pH gradients.

Software Analysis
Regardless of 2-DE or LC-based studies, any investigator is well advised to support the separation by software analysis. In particular, 2-DE is capable of delivering excellent quantitative data through specialized software. Software-assisted analysis of 2-DE spots is essential for comparative studies. Less than a decade ago, differences in intensities of the same spot between 2 sets of gels smaller than factor 2 were mutually considered too small and potentially reflecting methodical variance by proteomics reviewers. However, with the advent of DIGE and use of an internal pooled standard, an increasing number of reviewers started accepting articles reporting differences smaller than factor 2 in 2-DE-based proteome analyses (Table). Currently, a trend toward providing full analyses for a successful proteome analysis: highest resolution for separation is achieved under denaturing conditions. As a consequence, functional protein analyses currently require parallel processing of samples under non-denaturing conditions. Multiple affinity tag-based purifications have been developed to identify protein–protein interactions, but rarely reveal additional functional data, such as enzyme or signaling activity.

Future developments in proteomics endeavor non-denaturing analyses of proteomes to provide evidence for proteins being functional rather than expressed. As an example gaining attention, because of its emerging role in the cause of cardiovascular disease, the following paragraph focuses on the ubiquitin-proteasome system. It has been extensively studied by proteomics. However, only a limited number of studies use non-denaturing approaches to assess the intact structure of proteasome complexes (Figure). Notably, alterations in the abundance of proteasome subunits did not properly reflect their assembly into complexes during cardiac remodeling, as incorporation remained unchanged for some subunits, whereas it was delayed by several days for others. The selective incorporation rate of proteasome subunits in the pathogenesis of cardiac disease is of particular interest, because the myocardium harbors a heterogenic group of proteasome subpopulations. The discovery of cardiac proteasome heterogeneity under non-denaturing conditions was enabled by multidimensional LC supported by 2-DE (Table) as well as a novel approach for isoelectric focusing of multiprotein complexes in a laminar flow (free flow electrophoresis). Throughout non-denaturing sample preparation, function and assembly are the most suitable parameters for optimization and standardization.

Altogether, it is currently feasible to apply proteomics approaches, such as 2-DE, downstream of non-denaturing analyses in an effort to gain novel insights of structure (assembly) to function relationships. Current developments are directed toward increasing throughput as well as compatibility with other proteomics technologies.

Conclusions
Both 2-DE and LC approaches have fundamentally contributed to our understanding of cardiovascular diseases. Particularly, their power to analyze intact proteins at high throughput ensures their competitiveness for years to come. In cardiovascular research, many contractile or signaling proteins require post-translational processing to be functional and exist in multiple forms. By analyzing intact proteins, 2-DE and LC visualize heterogeneity and thus complete MS-based proteomics in the current trend to link PTMs to the pathogenesis of cardiovascular disease. Furthermore, 2-D DIGE is the method of choice when it comes to quantitative comparisons of intact proteins at large scale. Analyzing subproteomes, such as mitochondria or protein complexes, largely reduces the complexity of samples and navigates around the current limitations in proteomics. Combining proteomics with non-denaturing approaches will provide functionally validated targets for therapeutic approaches. Until non-denaturing proteomics is available for high throughput routine analyses in future, approaches focusing on intact proteomes can assist in either validating the identification or revealing differences potentially due to PTMs.
proteins or functional subproteomes will provide most revealing results about in vivo proteomes.

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