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Transfusion science: Integration of proteomics with blood banking

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Abstract

Blood banking comprises a constellation of activities from blood collection to testing and processing into individual blood components for transfusion purposes in which we seek to ensure the highest possible quality and safety. Challenged by the never-ending concerns and optimization endeavours of blood bank practice, research in transfusion medicine is currently tailored towards analyses of a variety aspect and issues spanning from donor to recipient. Over the last few years, proteomics technologies have been proven to be a valuable tool in transfusion research to tackle some of the unanswered questions in transfusion medicine. These studies include the storage lesions of platelet and red cell concentrates, changes in the platelet proteome during treatment with pathogen reduction technologies and quality assessment of plasma products. This review summarizes the current status of proteomic applications to blood banking and provides a perspective of the future.

Key words: Proteomics, blood banking, blood components.

Resumen

El trabajo en los bancos de sangre comprende una gama de actividades desde la donación sanguínea hasta la validación y obtención de componentes con fines terapéuticos, tratando de alcanzar la máxima calidad y seguridad. La investigación en medicina transfusional, desafiada siempre por las interminables preocupaciones y esfuerzos por optimizar las prácticas de los bancos de sangre, está enfocada actualmente hacia el análisis de una serie de aspectos y resultados que van del donador al receptor. En los años recientes, la tecnología proteómica ha demostrado ser una valiosa herramienta en la investigación transfusional para abordar algunas de las preguntas sin respuesta en medicina transfusional. Esto incluye estudios sobre las lesiones de almacenamiento de las plaquetas y concentrados de eritrocitos, cambios en el proteoma de las plaquetas durante el tratamiento con las tecnologías de reducción de agentes patógenos y la evaluación de calidad de los productos del plasma. Esta revisión resume el estado actual de las aplicaciones de la proteómica en los bancos de sangre y proporciona una perspectiva del futuro.

Palabras clave: Proteómica, bancos de sangre, componentes sanguíneos.

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1. Science in transfusion medicine

Transfusion medicine is the branch of medicine that is concerned with the transfusion of blood and blood components. Provided by national blood banks and under the supervision of physicians with transfusion medicine expertise, the quality and safety of blood and blood products are of highest priority. In order to fulfill this mission, research efforts are targeted to tackle questions concerning quality and safety. Projects carried out in either individual laboratories or in national or international collaborations such as the BEST Collaborative are tailored towards gaining a better understanding of the biology of blood and blood products or practises in the context of transfusion medicine covering all aspects from donor to recipient, hence «vein to vein».

Several scientific disciplines have joined to shed light into current issues in transfusion medicine; these range from biochemistry, molecular biology and physiology to physics, mathematics and bioengineering. In recent years, mass-spectrometry based proteomics has become a novel tool to approach questions in transfusion medicine from a protein level point of view. Placed in context with biochemical analyses, several of these analyses have been proven to be extremely useful to improve our view on blood product quality and safety. This review will provide a brief overview of the proteomics technology and its application to blood banking.

2. Mass-spectrometry-based proteomics

This chapter will briefly highlight the basics of mass spectrometry-based proteomics necessary to understand experimental design, data interpretation and the current challenges of this technology. Proteomics is the large-scale study of proteins expressed by a genome, cell, tissue or organisms, which is in general termed the proteome.[1,2] The protein profile can change with time, changes in the environment or specific treatments as part of the normal responses of living organisms. The word «proteome» is a blend of «protein» and «genome» and was coined by Marc Wilkins in 1994.[3] The term «proteomics» was first introduced in 1997[4] in analogy to genomics.

The quality and complexity of the sample or analyte directly influences the quality of the resulting data hence the type of sample determines the choice of mass-spectrometry (MS) technology. The mass spectrometer consists of three parts, the ion source, the mass analyzer that measures the mass to charge ratio (m/z) of the ionized analytes and the detector that registers the number of ions at each m/z value. Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are the two techniques most commonly used to volatize and ionize the proteins or peptides for mass spectrometric analyses. There are four basic types of mass analyzers used in proteomic research: ion trap, time-of-flight (TOF), quadrupole and Fourier transform ion cyclotron (FT-MS) analyzers. They are very different in design and performance and may be used alone or be combined to improve sensitivity, mass accuracy and resolution.

No proteomic method or mass spectrometric instrumentation exists that can identify components of a complex protein sample in a single-step.[5] Depending on the scientific question, combinations of individual techniques for separation, identification and quantification of (poly)peptides as well as tools for integrating and analyzing all the data are used. Over the last decade, two main tracks developed: protein-centric and peptide-centric. For the first approach, the proteins are separated by 1- or 2-dimensional gel-electrophoresis (1D/2D), stained and each observed protein spot is quantified by its staining intensity using sophisticated scanner/software systems. Selected spots are excised, digested and extracted peptides are

analyzed usually by MALDI-TOF or ESI-MS/MS systems. Although this approach was widely applied, it has become evident that this strategy suffers from a limited dynamic range.[6] However, incremental improvements in the 2D technology, including more sensitive or quantitative staining methods, large-format higher resolution gels and sample fractionation prior to electrophoresis have partially alleviated the shortcomings of the protein-centric approach. The alternative strategy, a peptide-centric approach compensates for some limitations of the protein-centric version. For example, an analysis of changes in plasma proteins over a period of storage in the thawed state may be readily undertaken using 2D technology; however, analysis of proteins in stored platelets may require peptide-centric approaches in order to capture more comprehensive information concerning membrane proteins than can be obtained by 2D technology alone. Importantly, peptide separation devices are used in multidimensional arrangements combining strong cation —affinity— and reverse phase chromatography methods to measure lower abundance proteins. To quantitate peptide LC-MS/MS experiments, stable-isotope labeling can be applied. This method makes use of the fact that pairs of chemically identical analytes of different stable-isotope compositions can be differentiated by spectrometry owing to their mass difference along with their relative proportion in the sample. To this end, methods such as isotope-tagging for relative and absolute quantitation (iTRAQ), isotope-coded affinity tagging (ICAT) and stable isotope labelling by amino acids in cell culture (SILAC) have been introduced.

From a biological point of view, the information about the amount or modification of an individual protein species is of high interest since implies alteration in protein or cellular function. During maturation, proteins undergo splicing, targeted degradation or post-translational modification (PTM). Recently, modification-specific enrichment techniques combined with advanced MS/MS methods and computational data analysis have revealed extensive PTMs in proteins, including multi-site, cooperative modifications in individual proteins.[7]

3. Analyses of (sub) proteomes of blood components

With the progress in analytical chemistry, the stage was set for the application of this technology to biological questions in blood. The initial target of investigations was plasma, in part because of the opportunity to study potential disease markers, and also because the lack of cells made sample preparation more straight forward. As discussed below, numerous extensive studies of the human plasma proteome have been undertaken, although relatively little of this information has been yet applied to transfusion medicine. The volume of literature on general proteomic studies of red cells and platelets is far less than plasma. Furthermore, more direct studies of stored blood products have begun to appear in the literature and an entire special issue devoted to blood proteomics was published in the *Journal of Proteomics* in January 2010. It contains a number of excellent and detailed reviews of direct relevance to transfusion medicine. The application of proteomics tools begins as a cataloguing exercise in which large amounts of data are acquired. The subsequent steps are the more relevant: the validation of the proteomic screening, the generation of testable biological hypotheses from the observed changes in protein amount or character, and the specific testing of those hypotheses.

In brief summary, the application of proteomics in transfusion medicine[8,9] has lead to several studies dealing with the systematic in-depth analysis of the protein content of various blood products,[10] such as plasma,[11] red blood cells,[12] as well as isolated, but never stored

platelets under resting conditions,[13-17] activated by thrombin receptor activation peptide (TRAP) or collagen.[18,19] To reduce the complexity of the proteomic sample, as well as improve assessment of low-abundance proteins, studies on platelet sub-proteomes such as the membrane,[20] microparticles,[21] α-granules,[22] and dense granules,[23] have been undertaken. Observations of changes in signaling proteins have since triggered analyses of the phosphoproteome under resting,[24] and activated conditions[18] as well as the determination of N-glycosylation sites on platelets.[25]

4. Proteomics for transfusion medicine

With the successful introduction of proteomics to transfusion medicine by the analysis of alterations of the proteomes of blood components, its application to quality and safety was proposed. At present, quality control of blood components is mainly focused on standardized quantitative assessment, providing relatively limited information about products. Unfortunately, during the production, inactivation, and storage processes there is the risk of changes, especially at the protein level, which could cause negative effects upon transfusion. It is therefore a major challenge to identify relevant alterations of these products, and, in this context, proteomics can play a potentially relevant role.[8,9]

4.1 Storage lesion of platelet concentrates

From a transfusion point of view, understanding the mechanisms that lead to the development of the storage lesion has been of longstanding interest. Storage-related changes in the pattern of cytosolic and membrane proteins were first observed in 1987 by Snyder and colleagues[26] using 2-dimensional gel electrophoresis (2D). They were only able to identify two actin fragments as significantly accumulating in platelets during the first 7 days of storage because of limits in genome sequencing and bioinformatics at that time. Although 2D possesses the disadvantage of being unable to adequately resolve large and extreme hydrophobic proteins as well as low abundance proteins due to its low dynamic range, it is on the other hand the only technique that allows for the separation of several thousand proteins at a time.[13] This is due to its large resolution capacity and increased loading ability using the zoom technology. The potential of proteomics as a viable tool for the elucidation of the PSL has increased dramatically with the development of mass spectrometry[10] and the subsequent development of quantitative proteomic techniques such as difference gel electrophoresis (DIGE) using highly sensitive fluorescence dyes leading to an improved dynamic range, as well as isotope-coded affinity tagging (ICAT), and isotope tagging for relative and absolute quantitation (iTRAQ).[27]

The DIGE technology was used to comprehensively assess the impact of storage on the global proteome profile of therapeutic platelet concentrates.[28] In order to overcome the disadvantages of the 2-DE technology as mentioned above, a complementary proteomic study was employed that addressed the relative differences using DIGE, ICAT, and iTRAQ in the analysis of the platelet storage lesion.[29] This strategy combines the power of the protein-centric approach characterized by high resolution achieved through the protein pre-separation, hints for changes in post-translational modifications as well as the detection of potential degradation. On the other hand, the peptide-centric approach compensates for the disadvantages of the gel system yielding a minimal protein loss and high dynamic range of protein analysis. The analysis of protein changes during platelet storage comprised the differences in the pattern between day 1 and day 7 and further analyses are necessary to determine more detailed information using a day-by-day

assessment because the actual levels of protein may be more dynamic and reflect the summation of *de novo* synthesis and degradation. *De novo* synthesis of proteins in anucleated platelets was discovered and analyzes in detail[30] revolutionizing platelet biochemistry. It has now been demonstrated for a variety of proteins.[31] During platelet storage, GPIIIa is translated throughout a 12-day period highlighting an amazing half-life of its mRNA of 2.4 days.[32] Recently, it was discovered that platelets produce progeny during *ex vivo* storage remaining some unanswered questions.[33]

Greening et al. have performed a comparison of human platelet membrane-cytoskeletal proteins with the plasma proteome.[34] This correlation provides the basis for the identification and classification of proteins that are selectively acquired from plasma by platelets such as L-lactate dehydrogenase, serum albumin, fibrinogen, carbonic anhydrase, endoplasmin, and multimerin 1 from those that are endogenous to platelets such as actin, actinin, filamin, tropomyosin, thrombospondin-1, platelet basic protein, platelet factor 4, and stomatin which are potentially released into the circulation or made available for concentrated and focal release at vascular sites of injury.

Lastly, diabetic patients are reported to have hyper-reactive platelets with exaggerated adhesion, aggregation and thrombin generation suggesting that their coagulation cascade is broadly dysfunctional.[35] A recent mass spectrometry based proteomics study was performed on platelets collected from healthy and type-2 diabetics and stored for transfusion.[36] This proteomic approach identified several proteins that were either up- or down-regulated in type-2 diabetics relative to non-diabetic controls or some proteins whose abundance changed during a 5-day storage period. Some of the proteins found changing confirmed the observations of the complementary proteomic approach[29] mentioned above including septin, superoxide dismutase, Rho-GDP dissociation inhibitor, and zyxin, as well as enzymes involved in glucose metabolism such as glucose-6-phosphate dehydrogenase and hexokinase. It was suggested that these identified differences allow the formulation of new hypotheses and experimentation to improve clinical outcomes by targeting «high risk platelets» that were hypothesized to render platelet transfusion less effective or even unsafe.[36]

4.2 Storage lesion of platelet and red cell concentrates

Proteomics has been applied quite successfully to studies of erythrocyte biology, including specific red cell disorders such as sickle cell anemia. It has also been applied to the elucidation of *in vitro* changes to red blood cells during standard storage conditions in an effort to understand the basis for the development of the red cell storage lesion. The red cell storage lesion is characterized by the impairment of ATP generation, the loss of 2,3-diphosphoglycerate, morphologic changes resulting in a loss of red cell flexibility and the blebbing of oxidatively damaged membrane as microparticles. Known characteristics of stored red cells, including formation of microvesicles are obvious targets for proteomic analysis. Proteomic studies contributing to our understanding of the storage lesion include.[37-41] These have provided more thorough analysis of changes to band 3 that appear to be involved in triggering the recognition of senescent cells by naturally occurring antibodies.[38,39] This observation hampers the usefulness of band 3 as a biomarker for old and/or damaged erythrocytes. Furthermore, this analytic strategy has provided very interesting information concerning the movement of specific red cell proteins into microvesicles and the physiologically relevant differences between intact red cells and microvesicles that may contribute to adverse

transfusion effects in aging cells. In parallel with microvesicle formation which removes band 3 disproportionally from the erythrocyte, changes are seen that reflect degradation of cytoskeletal proteins, and movement of signal transduction proteins and metabolic proteins away from the cell membranes.[41] The proteomic data have permitted investigators to establish a set of testable hypotheses of the relationship between microvesicle formation, oxidative damage, and erythrocyte clearance, as well as generate supporting data for models that were generated using non-proteomics techniques.

4.3 Proteomics to assess plasma quality

Proteomic analysis of plasma proteins began before the term «proteomics» was coined with the groundbreaking work of the Andersons in the 1970s in the development of high resolution two-dimensional gel electrophoresis.[42,43] In the last decade, the implementation of affinity-based depletion or enrichment strategies facilitated a deeper exploration into the plasma proteome by reducing the challenging dynamic range of protein concentrations.[44] This development has facilitated the identification of biomarkers[45] for the identification of individuals at risk of developing certain diseases, for the detection of existing disease and for the monitoring of therapeutic interventions in patients under treatment and subsequent determination of prognosis. Proteomics offers similar opportunities for transfusion medicine in the analysis of clinically relevant components of transfused plasma or of the quality of fractionated plasma proteins.

Human plasma is potentially the single most informative sample that can be collected from an individual since it contains not only the plasma resident proteins, but also representatives of other differentiated sub-proteomes of organs and compartments. The need for a systematic analysis of human plasma is emphasized by the plasma proteome project initiated by the Human Proteome Organization (HUPO) in 2002 (see http://www.hupo.org/research/hppp/). Candidate proteomic biomarker discovery from human plasma holds incredible clinical potential; however, it also poses significant challenges. The main obstacles are the dynamic range of proteins within plasma which exceeds ten orders of magnitude and the fact that many potential biomarkers are likely present at lower protein abundances. For example, human serum albumin has an abundance of ~45 mg/mL compared to cytokines which occur at concentrations of pg/mL or lower.

Although plasma protein profiling has been widely applied in various areas of medicine, proteomic technologies have thus far rarely been applied to plasma products in transfusion medicine. The analysis of prothrombin complex concentrates (PCCs) was used as a model to evaluate to what extent these technologies can detect differences in blood-derived therapeutics beyond that of standard quality control tests.[46] Additional studies have reported on fractionated blood products including FVIII and FVIII/von Willebrand factor concentrates[47] and FIX concentrates.[48] These studies showed a surprising degree of difference between products from different manufacturers but revealed low batch to batch variability of single products. Furthermore, protein impurities such as complement factors, clusterin or vitronectin were identified. Given that clotting factor concentrates are standardized principally by activity, proteomic methods provide additional information on the amount of inactive forms of these clotting factors.[46] Even these limited studies show that proteomics allows the identification of potentially modified proteins in clotting factor concentrates as well as in the rapid identification of low abundance contaminants, both of which can have impact on product immunogenicity.

4.4 Proteomics to assess effects of PRTs on blood components

Collectively, *in vitro* studies clearly demonstrate an impact of the PRT on platelet transfusion efficacy and further analyses are necessary to unravel the biochemical processes triggered by this treatment in order to understand the reduction in platelet quality. Studies have shown that UV-B irradiation causes platelet aggregation by activation of integrin αIIbβ_3 on the platelet surface and enhancing fibrinogen binding sites triggered by protein kinase C (PKC) activation via oxygen radicals.[49] This finding was further investigated in a study using UV-C irradiation demonstrating that the agonist-induced platelet response that usually lead to αIIbβ_3 activation is not involved in UV-C-mediated αIIbβ_3 activation. Instead, UV-C exerts a direct effect on aIIbb$_3$ by modifying extracellular disulfide bonds regulating integrin conformation, surprisingly independent of signal transduction.[50] A recent proteomic study of the effects of UV-C irradiation PRT technology (Theraflex, Macopharma, Mouvaux, France), used DIGE to catalogue proteomic changes before and after treatment compared to UV-B and gamma irradiation.[51] Only two proteins, IQGAP2 and ERp72, were exclusively affected by UV-C irradiation with the latter finding consistent with the observation that irradiation can influence disulphate-bond formation and consequently may lead to integrin activation.

In a proteomic study carried out in our laboratory, we used large format 1D gel electrophoresis to analyze changes in the soluble platelet proteome treated with riboflavin/UV (Mirasol technology, CaridianBCT, USA).[52] Alterations observed over the storage period in the samples from the untreated study arm reflect the classical platelet storage lesion which has been mentioned above. We found 14 reproducible protein changes in the treated study arm which were mainly cytoskeletal proteins involved in the maintenance of the actin structure and in the regulation of its dynamics. For further biochemical analyses, we focused on the vasodilator-stimulated phosphoprotein (VASP), a key player in actin cytoskeleton rearrangement controlling the dynamic actin turnover at the barbed ends of actin filaments. Activation is achieved by phosphorylation of the major sites Ser-157 and Ser-239 triggered by the cAMP- and cGMP-dependent protein kinases, respectively. Strikingly, VASP Ser-239 phosphorylation correlates very well with platelet activation determined by P-selectin expression on the platelet surface suggesting a link between VASP activation and granule transport.

Proteomic approaches offer an interesting way to investigate the effects of manipulation of plasma intended for transfusion. For example, the effects of various pathogen reduction technologies on the protein profiles may highlight areas for specific attention. Analysis of methylene blue treated plasma revealed modifications to γ-fibrinogen, apolipoprotein A1 and transthyretin.[53] Also the effect of solvent/detergent (S/D)-treatment on pooled plasma has been investigated using gel based proteomics, which showed alterations in α1-antitrypsin, α1-antichymotrypsin and α2-antiplasmin.[54] Lyophilization is a manipulation that may also affect proteins. However, studies using quantitative proteomics to assess lyophilized plasma after 24 months of storage found no impact on the plasma proteome.[54] With the large experience in plasma proteomics, the available quantitative proteomic approaches should be applied to further assess protein changes in plasma induced by pathogen reduction technologies.

4.5 Proteomic studies with potential transfusion relevance

Other studies have been carried out which might have an impact on transfusion medicine. Based on the fact that the incidence of cardiovascular diseases is ten-times higher in males than in

females, computer simulation *(in silico)* and proteomic approaches were employed to analyze whether gender-specific differences exist in blood platelets since antiplatelet drugs have assumed center-stage for prevention and therapy of most types of CVD.[55] Gel-based proteomics and micro-array analyses have revealed that platelets from male donors express significantly higher levels of signaling cascade proteins than platelets from female donors indicating that this difference may suggest a biological mechanism for gender discrimination in cardiovascular disease. However, the impact of this finding on transfusion medicine is unknown at this point of time.

Deficiencies in granule-bound substances in platelets cause congenital bleeding disorders known as storage pool deficiencies. For disorders such as gray platelet syndrome (GPS), in which thrombocytopenia, enlarged platelets and a paucity of α-granules are observed, only the clinical and histologic states have been defined. As compared with control, the soluble, biosynthetic α-granule proteins were markedly decreased or undetected in GPS platelets, whereas the soluble, endocytosed or membrane-bound α-granule proteins were only moderately affected.[22] These results support the existence of «ghost granules» in GPS, point to the basic defect in GPS as failure to incorporate endogenously synthesized megakaryocytic proteins into α-granules, and identify specific new proteins as α-granule inhabitants.

In another study, platelets as well as their releasate after TRAP activation from patients with clinical bleeding problems evidenced for platelet storage pool disease were analyzed by DIGE-based proteomic technology.[56] A total of 44 differentially expressed proteins in patients were identified; most of these proteins belong to the class of cytoskeleton-related proteins. In addition, 29 cytoskeleton-related genes showed an altered expression in platelet mRNA from patients using a real-time PCR array. In conclusion, this study shows that the dense granule secretion defect in patients with platelet storage pool disease is highly heterogeneous with evidence of an underlying cytoskeleton defect.

5. Future perspective

The application of proteomics to transfusion medicine has allowed the detailed analyses of the proteome of blood components and subsequently provided new insights into alterations during blood processing and storage. Recent studies such as the investigation of the impact of pathogen reduction technologies on blood platelets or the analyses of the (sub)proteome of blood components from patient samples revealed potential molecular mechanisms that could cause the reduction in platelet quality or disease development, respectively. The continued expansion of proteomic applications in various areas of hematologic biology contributes to the generation of data that will define the path where proteomics in transfusion medicine will head in the near future. However, as mentioned earlier,[57] it important to recognize that this cannot be a simple cataloguing of protein changes. We also need to understand the relationship between the proteomic results and the molecular mechanisms of processes under study. With this concept firmly in mind, many unanswered questions as well as future challenges in transfusion medicine can be tackled spanning-from donor to recipient.

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