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# Mass Spectrometry-Based Analysis Of Membrane Proteins Derived From Chronic Lymphocytic Leukemia And Multiple Myeloma Cells

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## Abstract

Plasma membrane proteins play a key role in various cellular processes, such as cell-interactions, transport and signaling. Currently about 70% of all known drugs target membrane proteins; therefore a better characterization of the membrane proteome is essential for understanding their role in basic biological functions and for finding new drug targets. However, they are usually under-represented in proteome-datasets due to difficulties in extraction, digestion and separation. To enhance the identification of membrane proteins different separation and enrichment strategies were applied, including density gradient ultra-centrifugation and affinity-enrichment with Concanavalin A, plus several solubilization and digestion approaches using different detergents and proteolytic enzymes. The aim was to establish an appropriate procedure for the identification of membrane proteins for the comparative analysis of CLL (chronic lymphocytic leukemia) and MM (multiple myeloma) cells with mass spectrometry. From all the enrichment strategies mentioned above, density gradient ultracentrifugation performed best in identifying the largest number of membrane proteins and was applied for the comparative analysis of JVM-13 (CLL) and RPMI 8226 (MM) cells using a nano-flow LC orbitrap MS/MS. The proteins identified could easily be used to distinguish between the two cell lines (3897 significantly different proteins) and some were also be found in primary CLL and MM cells. In addition, some membrane proteins relevant in CLL and MM pathogenesis were exclusively identified with density gradient ultracentrifugation. These results indicate that density gradient ultracentrifugation remains the “golden-standard” when analyzing membrane proteins and that cell lines are well suited model systems for detailed studies of various diseases, including CLL and MM.

## Keywords:

Membrane proteins

Proteomics

Mass spectrometry

Multiple myeloma

Chronic lymphocytic leukemia

## 1. Introduction

Biological membranes form a protective barrier between the cell and its environment and serve to compartmentalize intracellular organelles in eukaryotic cells. Although the basic structure and function of cellular membranes are determined by the lipid bilayer, embedded proteins contribute to organ- and tissue-specific functions of the cell (Wu et al. 2003). Plasma membrane proteins regulate various cellular processes such as cell-cell and cell-matrix interactions, transport of ions and other molecules, as well as different defensive and signaling functions. Due to their biology, diversity and unique behavior during purification and separation, membrane proteins hold a special position in the field of proteomics (Josic et al. 2007). Although 20-30% of the human genome encodes for membrane proteins, they are under-represented in most proteome datasets (Tan et al. 2008). Some of the difficulties in connection with proteome analysis of membrane proteins involve their low abundance (in comparison with cytoplasmic proteins for example) and their hydrophobic nature.

Profiling the dynamic changes of the plasma membrane proteome is very important in understanding basic biological processes and pathological mechanisms in the cell, and for finding targets that may eventually enable disease diagnosis and intervention (Sprenger et al. 2010). Currently about 70% of all known drug targets are membrane proteins, with 25% being G-protein coupled receptors (GPCRs) alone. Identifying uniquely expressed surface proteins may also lead to the development of new therapeutic monoclonal antibodies, such as rituximab (anti-CD20 antibody) for the treatment of chronic lymphocytic leukemia (CLL) (Wu et al. 2003). CLL is a B-cell malignancy characterized by the accumulation of CD5+ B-lymphocytes in the peripheral blood and bone marrow ( $> 5 \times 10^9/l$ ). It is the most common leukemia in adults in the western world and is usually treated with a combination of fludarabine, cyclophosphamide and rituximab (FCR) (Eichhorst et al. 2010). Multiple myeloma (MM) is another form of B-cell non-Hodgkin lymphoma and characterized by clonal proliferation of plasma cells within the bone marrow. MM has the same incidence than CLL (4:100.000) and shares some other biological and clinical similarities. Biologically, both malignancies are associated with different precursor diseases (monoclonal gammopathy of undetermined significance in MM and monoclonal B-cell lymphocytosis in CLL) and affect older, mainly male, individuals older than 60 years (Landgren/ Kyle 2007). Common clinical features include stage-dependent anemia and immune deficiency, as well as late stage unresponsiveness to therapy. MM is, however, treated very differently from CLL, usually including high-dose chemotherapy (mostly bortezomib and dexamethasone combined with thalidomide, doxorubicin, lenalidomide or cyclophosphamide) followed by autologous stem-cell transplantation (Röllig et al. 2015). Furthermore, compelling evidence suggests that CLL and MM strongly depend on external stimuli from their tumor micro-environment, such as bone marrow and secondary lymphoid organs, promoting disease progression and drug resistance. Membrane proteins, which are in direct contact with the surrounding stromal cells, therefore play a key role in the supportive interactions with the tumor microenvironment (Burger et al. 2009). Comparing the membrane proteome of MM- and CLL-cells may lead to a better understanding of the similarities and the differences of these two diseases, especially concerning their different course of treatment.

## 2. Methods

The analyzed membrane proteins were obtained from commercially available MM (RPMI 8226) and CLL (JVM-13) cell lines ordered from the German Collection of Microorganisms and Cell Cultures. Preliminary tests to evaluate different membrane enrichment approaches were conducted using HCT 116 cells, which is a colon carcinoma cell line, purchased from ATCC. RPMI 8226 and JVM-13 cells grow in suspension and require RPMI medium, while the adherent HCT 116 cells need McCoy's medium. All three cell lines were cultivated with 10% FCS and 0.1% antibiotics at 37°C and 5% CO<sub>2</sub> until they reached a density of  $\sim 1 \cdot 10^6$  cells/ml or a confluence of 70-80%. To enhance the identification of membrane proteins in a complex matrix different enrichment and separation strategies were applied, including density gradient ultracentrifugation (Avanti™ J-301, Beckman Coulter®) and affinity enrichment using biotinylated Concanavalin A (Vector Labs®) coupled with streptavidin-agarose and streptavidin -magnetic beads (Thermo Fisher Scientific®). Density gradient ultracentrifugation of whole cell lysates is a routinely used method to isolate membrane proteins, in which the membranes are separated from other subcellular organelles due to their different densities. To improve the resolution and separation of this method, adding a sucrose gradient was also evaluated. In the second approach, membrane proteins were enriched because of their affinity towards lectins, in this case Concanavalin A (Con A). Lectins are carbohydrate-binding proteins that bind specific sugar moieties and can therefore be used to purify glycosylated membrane proteins (Lee et al. 2008).

To avoid under-representation of highly hydrophobic proteins and maximize their total yield, different solubilization and digestion approaches were also tested, including the use of different combinations of detergents (e.g. Triton X-100, N-decyl- $\beta$ -D-maltopyranoside, SDS and CHAPS), extra washing steps with varying concentrations of acetonitrile and/or acid as well as the application of another proteolytic enzyme. Following solubilization and protein digestion, the isolated membrane proteins were identified using nano-flow LC orbitrap MS/MS (Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer) and evaluated using database analysis (Proteome Discoverer 1.3 Software, Thermo Scientific™ and MaxQuant Software, 1.3.0.5). The most important setting for both database searches included a false discovery rate (FDR) of  $< 0.01$ , a maximum of two missed cleavages as well as carbamidomethylation on cysteins for fixed modifications and methionine oxidation or N-terminal protein acetylation for variable modifications. After the database search was completed, the acquired data was statistically evaluated using the Perseus Software 1.3.0.4 and a two-sided t-test ( $p < 0.05$ ) was performed to identify significantly up- and down-regulated proteins.

## 3. Results

Of all the membrane enrichment approaches evaluated, density gradient ultracentrifugation performed best in identifying most membrane proteins in HCT 116 cells. In one 280 min LC-MS/MS run 3117 overall protein identifications were achieved with 1583 membrane proteins, 407 plasma membrane proteins and 668 transmembrane proteins, according to Uniprot database. However, a fair number of

cytoplasmic (1259) and nuclear (1116) proteins were also found in the membrane fraction. In comparison, adding a sucrose gradient did not improve the identification of membrane proteins (1399 membrane proteins, 365 plasma membrane proteins and 500 transmembrane proteins) or decreased contamination with non-membrane proteins (1295 cytoplasmic and 1111 nuclear proteins). Affinity-purification via Concanavalin A also lacked in identifying more membrane proteins, with 1264 membrane proteins and only 322 plasma membrane proteins and 482 transmembrane proteins according to Uniprot database.

Based on the results of all preliminary tests, the comparative analysis of RPMI 8226 and JVM-13 was carried out using density gradient ultracentrifugation for the enrichment of membrane proteins that were digested using the standard protocol with an extra washing step with 1% FA. When compared to the traditional fractionation approach, density gradient ultracentrifugation was able to identify more membrane proteins, including more transmembrane- and plasma membrane proteins, in both cell lines. For RPMI 8226 4661 proteins were identified, of which 1975 were annotated to “membrane”, 561 to “plasma membrane” and 1295 to “transmembrane”. In JVM-13 4039 overall protein identifications were achieved, with 1608 membrane, 513 plasma membrane and 925 transmembrane proteins. Robustness and reproducibility was slightly better for the standard fractionation protocol when compared to ultracentrifugation, but the correlation coefficient  $r^2$  was not lower than 0.829 for the biological replicates in the ultracentrifugation approach. When further comparing the two fractionation strategies, some proteins were only identified with the standard protocol and some were only found in the ultracentrifugation approach. Examples of membrane proteins only identified in the ultracentrifugation method are tumor necrosis factor receptor superfamily member 17 for RPMI 8226 and CD180 for JVM-13 cells. Moreover, we were able to distinguish between the two cell lines via differentially expressed surface markers characteristic for MM- or CLL-cells: CD138, CD38, CD28 and CD33 for RPMI 8226 and CD19, CD20, CD23 and CD79 A& B for JVM-13. In addition to the cells’ differing immunophenotype, there were also several other proteins identified relevant to disease development and progression in MM and CLL. For example, the insulin-like growth factor 1 receptor, the interleukin-6 receptor subunit beta or osteopontin for MM and tyrosine-protein kinase ZAP-70, tyrosine-protein kinase BTK or T-cell leukemia/lymphoma protein 1A for CLL.

#### 4. Discussion

The aim of this master thesis was to establish an appropriate procedure for the identification of membrane proteins for the comparative analysis of CLL- and MM-cells with mass spectrometry. Various strategies have been tested to improve the solubilization, separation and identification of hydrophobic peptides in a proteomic workflow. In this master thesis, density gradient ultracentrifugation was identified as the so-called “golden-standard” approach for the enrichment of biological membranes and membrane proteins. No other purification approach worked as well in identifying as many membrane proteins, including transmembrane proteins, according to Uniprot database. In RPMI 8226 cells, almost 2000 membrane proteins could be identified, including 561

plasma membrane and 1295 transmembrane proteins. In comparison, Mann et al. identified 2223 membrane proteins and 2040 transmembrane proteins out of 3528 overall identifications in rat cerebellum tissue (Lu et al. 2009). It must be stressed, however, that they used 4-5mg of protein for their analysis as opposed to 25µg that were obtained by ultracentrifugation protocol of two 75cm<sup>2</sup> cell culture flasks (1,54 x10<sup>6</sup> cells/ml). Moreover, brain tissue is very rich in membrane proteins when compared to lymphocytes or epithelial cells.

In addition to the high amount of membrane proteins identified, some proteins only found in the ultracentrifugation fraction are crucial in MM and CLL pathogenesis, for example, the ABC transporter G family member 2 (ABCG2) for MM and the interleukin-21 receptor for CLL. Furthermore, some proteins not yet reported to be involved in CLL or MM have been found with this novel approach, e.g. Proline-, glutamic acid- and leucine-rich protein 1 or N-acetyltransferase 10. This underlines the importance of density gradient ultracentrifugation or other membrane enrichment strategies when analyzing the membrane proteome. Some of the proteins mentioned above were also identified in primary MM and CLL cells, emphasizing the importance and suitability of tumor cell lines for the study of various diseases, including cancer.

In conclusion, proteome profiling proved to be successful in identifying all known characteristic membrane proteins of CLL and MM cells. Furthermore, several previously unrecognized proteins were identified, which may help to better understand the function of these neoplastic cells.

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