Proteomics

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# Proteomic Analysis of Plasma Membrane Vesicles**

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Micrometer-sized plasma membrane vesicles (PMV) are released from the cell surface upon exposure to formaldehyde (FA) and dithiothreitol (DTT). This phenomenon was recognized 30 years ago,[1–3] and the formation mechanism ("blebbing"), and types of PMVs and formation methods have been investigated to some degree.[4–6] It is believed that PMVs are formed owing to weakening of the bonds between the cytoskeleton and the plasma membrane followed by volume expansion driven by intracellular pressure.[4,7,8] PMVs are used in a number of applications, for example, as a source of membrane and protein material for constructing vesicle–nanotube networks,[9] and as model systems for studying phase separation of proteins and lipids.[10] Investigations on vesicle biogenesis,[11] the structure of the IgE receptor by FRET,[12,13] lateral diffusion of membrane proteins,[14] and distribution of GPI anchored proteins[15] have also been performed. It has also been shown that PMVs can be obtained from excised endothelial tissue,[16] further expanding application areas beyond the use of cell cultures. It is presumed that plasma membrane (PM) proteins and lipids are extracted during vesicle formation, and thus are uniformly present in the PMV membrane, whereas the interior is filled with cytosol but lacks cytoskeletal structures. However, no membrane proteomic characterization has been performed on these types of FA/DTT-generated vesicles.

There are, however, several reports of proteomic analyses of various other types of cell-derived vesicles, for example, secretory vesicles,[17,18] clathrin-coated vesicles,[19] exosomes,[20,21] and membrane microparticles (MMP).[22] Membrane microparticles are formed, for example, after stimulation with apoptotic or mitogenic stresses,[23] and their rich membrane proteome has been well-characterized in lymphocyte membranes.[22]

Herein we develop a method for harvesting, purification, and processing of PMVs recovered from the NG108-15 cell line, with subsequent analysis of the membrane proteome after solid-phase proteolytic digestion in a microfluidic flowcell, and LC-MS/MS analysis of the resulting peptides. The method is highly flexible in terms of digestion, labeling, and derivatization, owing to the on-flowcell immobilization of the sample. For example, fluorescent labels can be introduced, or various alkylation and reduction reactions can be performed. The on-flowcell method can presumably be applied to other types of cell-derived vesicles as well. A total of 313 proteins were identified, of which 43 could be annotated to the plasma membrane, another 49 were tentatively membrane-associated, and the rest were of cytosolic origin. A total of 93% of the membrane proteins were derived uniquely from the plasma membrane pool.

To produce and purify PMVs in high yields, NG108-15 cells are grown to circa 80% confluency to obtain about $15 \times 10^6$ cells. The cell layer is then thoroughly washed with a buffer solution to completely remove the culture medium, as serum proteins would present a source of contamination in proteomic analysis. Vesiculation is then induced by adding vesiculation solution containing dithiothreitol (DTT) and formaldehyde (FA) directly to the culture flask (Figure 1A,B). This formulation is known to produce PMVs in 23 different cell types.[2,3,12–14,16,24,25]

It has been observed that a single cell can shed up to ten vesicles.[3] According to our observations, a single NG108-15 cell could produce three 10 μm-diameter PMVs in a time window of 2 h. This amounts to about 300 $\mu m^2$ membrane area released from the PM of a single cell.

To estimate the increase of PMV yield at longer incubation times, PMV production from a confluent 25 $cm^2$ flask of WSS1 cells was observed using a Buerker cell counting chamber. For this, samples were taken every hour and the yield was calculated (Figure 2). Assuming a mean PMV diameter of 5 μm, circa 15 $cm^2$ membrane can be recovered from a confluent 75 $cm^2$ flask. After one hour of incubation, $1.83 \times 10^6$ PMVs with an average diameter of 5 μm were already produced, and after 8 h of incubation, $1.96 \times 10^7$ PMVs had been produced, that is, a tenfold increase in total PMV yield.

When estimating the PMV yield of CHO-K1 cells, it was noted that the CHO-K1 cell line produced less membrane material. This can be explained by the fact that CHO cells are of smaller size, and thus may possess less excess membrane material for PMV production. Membrane proteins are present even in later PMV generations, as glycoproteins can be readily detected by labeling using a wheat germ agglutinin-conjugated dye (data not shown).

We also estimated the rate of membrane release by observing the growth time of one cell-attached PMV at room temperature. Assuming the production of 3 PMVs, an expansion by 5 μm in diameter (5 μm→10 μm) in 30 minutes corresponds to a membrane release rate of about 8 $\mu m^2 min^{-1}$ per cell. For comparison, endocytosis rates are circa

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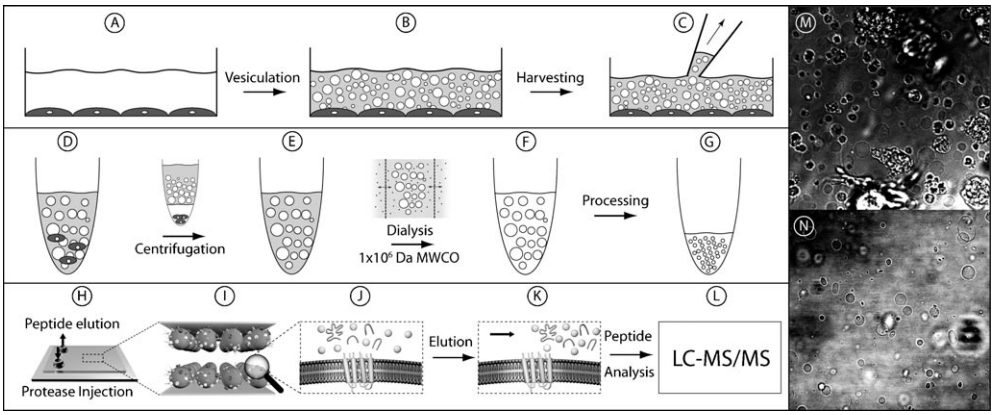


***Figure 1.*** Formation, purification, and proteomic analysis of PMVs. A) A confluent cell layer is washed and incubated with vesiculation solution. B) During incubation, PMVs are formed at the cell surface and bud off into the solution. C) The PMV solution is carefully aspirated from the cell layer. D), E) The harvested PMV solution is underlaid with a 2 M sucrose solution with subsequent low-speed centrifugation. E), F) The upper phase is collected and dialyzed against HEPES buffer using a large cutoff dialysis membrane. F), G) The purified PMV solution is prepared for subsequent proteomic analysis. PMVs are exposed to reducing and alkylating agents, sonicated, and washed to further remove soluble proteins. H), I) Processed PMVs are immobilized on the flowcell surface by injecting the PMV solution with the inlet nozzle. J), K) After injection of protease, surface-exposed domains of membrane proteins are cleaved and peptides are eluted from the chip by the outlet nozzle. L) The eluted peptide sample is processed and analyzed by LC-MS/MS. M) Differential interference contrast microscopy (DIC) image of a crude PMV solution corresponding to (D). N) DIC image of a purified PMV solution after dialysis, corresponding to (F).

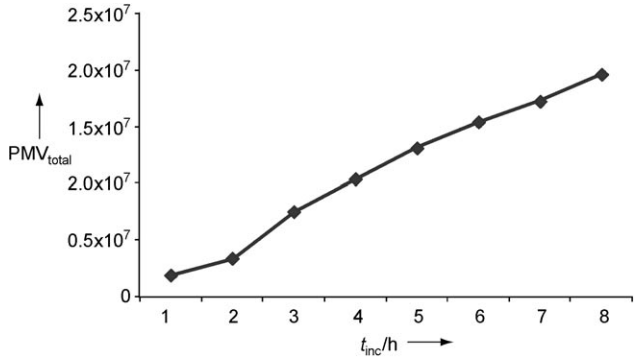


***Figure 2.*** Number of PMVs ($PMV_{total}$) produced by the WSS1 cell line as a function of incubation time $t_{inc}$.

5 $\mu m^2 min^{-1}$ per cell.[26] It can be speculated that a cell can release even more PMVs over a longer incubation period, as after removal of the first PMV generation (ca. 12 h), an additional incubation round of 12–24 h, using fresh vesiculation solution, still yields a large amount of PMVs. However, the third generation of PMVs, which was harvested 60 h after the first incubation round, had a considerably smaller mean diameter, indicating the depletion of available membrane stores.

After vesiculation, the PMV-containing solution has to be purified as it contains a range of substances which contaminate proteomic analysis. First, PMVs have to be separated from other membranous particles, such as detached cells, and cell debris. As PMVs are filled with cytosol and have a similar size as cells, a large fraction will pellet together with cellular material during centrifugation, hindering effective separation. To avoid this problem, we utilized the difference in density of PMVs compared to cells. The PMV solution is carefully aspirated (Figure 1 C), transferred into a centrifuge tube, underlaid with a high-density sucrose phase, and centrifuged. PMVs accumulate at, but do not cross, the buffer/sucrose phase boundary, whereas cells pellet at the bottom of the tube, ensuing an almost complete separation of cells and PMVs (Figure 1 D, E). An alternative method to separate PMVs and cells is to subject the sample to a freeze–thaw cycle. The sample is snap frozen in liquid $N_2$ and thawed at 37 °C. This procedure is repeated four times, resulting in fragmentation of PMVs to less than 1 μm, simplifying purification by differential centrifugation. It should be noted that soluble proteins from the interior of cells are likely to be present in the sample.

Additional contaminating material is soluble protein that is supposedly released from cells during the vesiculation procedure and from collapsed PMVs. The vesiculation agent FA will also hamper the efficiency of the downstream protease digestion owing to its protein crosslinking activity. The next important step therefore is efficient dialysis of the sample against buffer solution, for which we use a dialysis membrane with a very high cutoff (1 MDa) to remove as much interfering material as possible together with vesiculation agents (Figure 1 E, F).

After dialysis, the PMV sample undergoes a final processing step before digestion is performed (Figure 1 G). First, surface-exposed membrane proteins are reduced with DTT and alkylated with iodoacetamide to break and protect disulfide bonds, respectively, making more cleavage sites available for digestion and to prevent protein aggregation. Second, a washing step at high pH values disrupts noncovalent protein–protein interactions, dissociating cytosolic proteins from the membrane. Presumably, this treatment also causes PMVs to disrupt,[27] and consequently, together with extensive sonication, the cytosolic interior is released into the PMV solution. To remove this additional contamination source, the PMV membranes are pelleted by ultracentrifugation and the supernatant is removed. Finally, the membrane pellet is rinsed and dispersed by sonication in ammonium bicarbonate buffer and is ready for digestion.

Digestion is performed in-solution, as well as after PMV-immobilization in a flowcell. For in-solution digestion, trypsin is added to the processed PMV solution, and the peptides are

  

separated from the membranes by low-cutoff filtering. This method is fast and simple, but has the disadvantage that cytosolic proteins in the sample may cause clogging of the LC columns in subsequent analyses, and that it is difficult to maintain the membrane protein fraction. The latter method uses the lipid-based protein immobilization (LPI) flowcell technology. The working principle of the flow cell is based on solid-phase immobilization of PMVs, allowing for simple buffer/reagent exchange, and sample handling (Figure 1H–L). The PMV solution is injected into the flowcell, where membranes and also proteins adhere to the surface. Injection of the trypsin solution initiates digestion of protein domains, which are exposed on the surface of immobilized PMVs (Figure 1J). As some soluble protein contaminants are immobilized and many are washed out during repeated washing cycles without sacrificing the membrane protein fraction, the flowcell also provides a purification step yielding clean peptide fractions. Finally, the peptides are eluted and analyzed by LC-MS/MS.

To determine the subcellular origin of the PMV membrane, we investigated the subcellular location of the membrane proteins found therein. Five independent PMV samples were analyzed, resulting in a total of 313 protein identifications. According to the sources we use to annotate membrane association and subcellular location, 43 PMV proteins (14%) are anchored to the membrane by at least one α-helical domain or a lipid anchor (Supporting Information, Table S1), and 49 (16%) are associated with the membrane by other interactions. 40 of the anchored membrane proteins are located to the PM (93%), of which 32 proteins (74%) are unique to the PM (Figure 3). For the remaining 221 proteins we could not identify any membrane association, and presume that these are soluble proteins originating from inside PMVs.

Among the identified membrane proteins, GTPases and G-Proteins are predominantly found. Amino acid transporters, ion transporters, and proteins responsible for cell adhesion and growth are also represented (Figure 4). Notably,

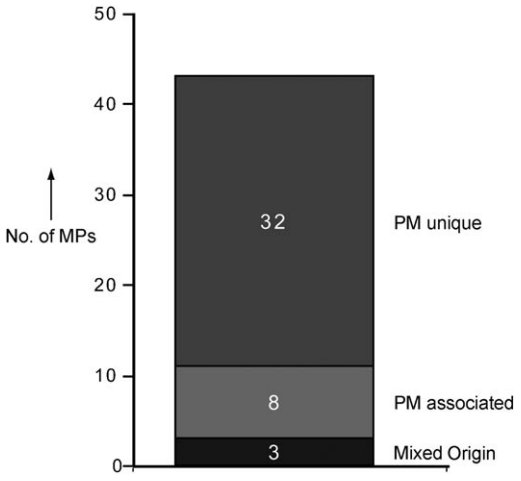


***Figure 3.*** Identified membrane proteins in purified PMVs. 40 out of 43 membrane proteins are annotated to the PM (i.e.93%). Of these, 32 membrane proteins are uniquely located to the plasma membrane.

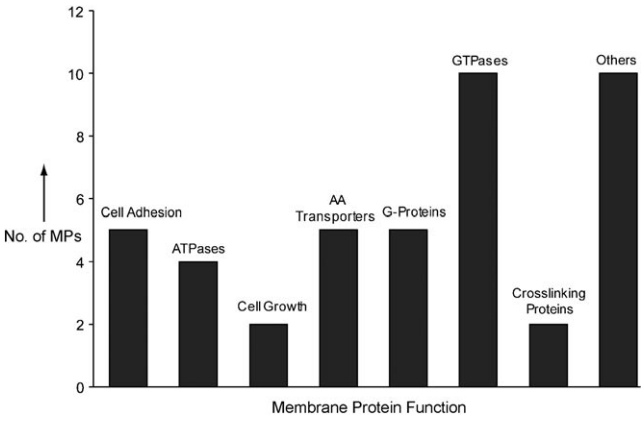


***Figure 4.*** Classes of PM proteins found in PMVs.

putative plasma membrane-cytoskeletal crosslinking proteins were also identified, indicating that the vesiculation process might cause dissociation of these proteins from the cytoskeleton. It is possible that highly hydrophobic integral membrane proteins that do not have large trypsin-cleavable loops outside the membrane are missed using the current digestion technique. Furthermore, reversing the crosslinking effect of formaldehyde[28] might help to optimize tryptic cleavage, and could result in a greater number of identified (membrane) proteins. However, the most important improvement to the method would be to optimize downstream analysis of the yielded peptides. For example, 1D-SDS-PAGE in combination with LC-MS/MS or even 2D-LC-MS/MS would most likely yield much more data than presently achieved.

In our PMV analyses, we could identify 221 soluble proteins, many of them ribosomal and cytosolic, which originate from the PMV interior. Presumably, these are released during the processing steps after dialysis of the PMV sample. The first sonication step causes the micrometer-sized PMVs to disrupt and reseal, releasing the cytosolic interior. Ribosomes appear to be abundant in the PMV solution, and owing to their large size, they cannot be removed by dialysis and are likely to be pelleted together with the processed PMV membranes in the ultracentrifugation step. Furthermore, as the membrane pellet undergoes an additional sonication step just prior to digestion, additional release of cytosolic proteins might occur, which could add to the soluble protein count in the obtained result. Further optimization, including fine-tuning of centrifugation steps to remove ribosomal proteins, or finding alternatives to the last sonication step, might bring these contamination sources down to a minimum. However, it should be noted that a proteomic analysis of the PMV interior can be extremely useful for other purposes, such as investigation of membrane protein activities coupled with cytosolic proteins, which go beyond the focus of this article.

It appears that membrane proteomic methods based on analyzing the plasma membrane protein content directly from vesicles yield much higher purity[22] than traditional methods for analyzing mammalian PM proteomes.[29–34] The difficulties in membrane proteome analysis arise primarily from the presence of membrane proteins in subcellular compartments and in the plasma membrane. Many membrane proteins are

  

therefore assigned to multiple cellular locations, as was observed by protein correlation profiling.[35] Various methods have been developed to isolate PMs for proteomic studies,[36–38] for example, affinity enrichment,[39–41] but what all these methods share in common is the problem of contamination, in which other organelles still account for 30–40 % of identified membrane proteins, hampering identification of unique PM proteins.[40,42–44]

Besides by formation of PMVs, cells can also release plasma membrane in the form of microparticles. It is therefore interesting to highlight some of the main differences between these two vesicle types. Microparticles are shed upon a number of stress conditions, including apoptosis.[22] Such stimulation induces a cascade of cellular processes whereby the proteome of microparticles is believed to depict a different membrane protein composition compared to a "resting" cell.[22] Formation of PMVs occurs upon exposure to sulfhydryl blocking agents and requires calcium ions.[45] Physically, PMVs are larger than microparticles (5–10 μm compared to 0.1–1 μm in diameter), thus they are more "planar" (larger radii of curvature), allowing the presence of large aggregate structures including lipid rafts. A number of G-proteins, which are generally known to preferentially segregate in lipid rafts, are found in the PMV proteome, which backs up a previous study where lipid rafts were readily visualized in PMVs.[10] Furthermore, in the total PMV proteome only 3 out of 313 detected proteins (less than 1 %) were found to derive from intracellular membranes, whereas the microparticle proteome includes about 10 %. The similarities in the PMV and microparticles proteome are the comparatively large amount of identified cytoplasmic proteins and the overall very high purity in regard to PM proteins in the membrane proteome. PMVs are produced over a quite long time span, thus by analyzing different PMV generations, it may be possible to study if internal membrane stores are recruited for PMV formation after prolonged incubation time (more than 24 h). As the PMVs are micrometer-sized, they are suitable for microscopic investigations that can yield important complementary information to proteomic analysis, for example, lipid raft formation, and phase behavior.[10]

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[1] R. E. Scott, *Science* **1976**, *194*, 743.
[2] R. E. Scott, P. B. Maercklein, *J. Cell Sci.* **1979**, *35*, 245.
[3] R. E. Scott, R. G. Perkins, M. A. Zschunke, B. J. Hoerl, P. B. Maercklein, *J. Cell Sci.* **1979**, *35*, 229.
[4] G. T. Charras, J. C. Yarrow, M. A. Horton, L. Mahadevan, T. J. Mitchison, *Nature* **2005**, *435*, 365.
[5] M. M. B. D. D. J. Hagmann, *J. Cell. Biochem.* **1999**, *73*, 488.
[6] H. Keller, P. Rentsch, J. Hagmann, *Exp Cell Res.* **2002**, *277*, 161.
[7] C. C. Cunningham, *J. Cell Biol.* **1995**, *129*, 1589.
[8] A. Boulbitch, R. Simson, D. A. Simson, R. Merkel, W. Hackl, M. Barmann, E. Sackmann, *Phys. Rev. E* **2000**, *62*, 3974.
[9] B. Bauer, M. Davidson, O. Orwar, *Langmuir* **2006**, *22*, 9329.
[10] T. Baumgart, A. T. Hammond, P. Sengupta, S. T. Hess, D. A. Holowka, B. A. Baird, W. W. Webb, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 3165.
[11] E. E. Golub, S. C. Schattschneider, P. Berthold, A. Burke, I. M. Shapiro, *J. Biol. Chem.* **1983**, *258*, 616.
[12] D. Holowka, B. Baird, *Biochemistry* **1983**, *22*, 3466.
[13] D. Holowka, B. Baird, *J. Biol. Chem.* **1984**, *259*, 3720.
[14] D. W. Tank, E. S. Wu, W. W. Webb, *J. Cell Biol.* **1982**, *92*, 207.
[15] N. A. Baumann, J. Vidugiriene, C. E. Machamer, A. K. Menon, *J. Biol. Chem.* **2000**, *275*, 7378.
[16] N. I. Moldovan, A. N. Radu, N. Simionescu, *Exp. Cell Res.* **1987**, *170*, 499.
[17] D. Jethwaney, M. R. Islam, K. G. Leidal, D. B. V. de Bernabe, K. P. Campbell, W. M. Nauseef, B. W. Gibson, *Proteome Sci.* **2007**, *5*, 12.
[18] J. Wegrzyn, J. Lee, J. M. Neveu, W. S. Lane, V. Hook, *J. Proteome Res.* **2007**, *6*, 1652.
[19] G. H. H. Borner, M. Harbour, S. Hester, K. S. Lilley, M. S. Robinson, *J. Cell Biol.* **2006**, *175*, 571.
[20] R. M. Johnstone, *Blood Cells Mol. Dis.* **2006**, *36*, 315.
[21] R. J. Simpson, S. S. Jensen, J. W. Lim, *Proteomics* **2008**, *8*, 4083.
[22] L. Miguet, K. Pacaud, C. Felden, B. Hugel, M. C. Martinez, J. M. Freyssinet, R. Herbrecht, N. Potier, A. van Dorsselaer, L. Mauvieux, *Proteomics* **2006**, *6*, 153.
[23] B. Hugel, M. Carmen, M. C. Martinez, C. Kunzelmann, J. M. Freyssinet, *Physiology* **2005**, *20*, 22.
[24] R. E. Scott, A. S. Rosenthal, *J. Immunol.* **1977**, *119*, 143.
[25] P. Sengupta, A. Hammond, D. Holowka, B. Baird, *Biochim. Biophys. Acta* **2008**, *1778*, 20.
[26] P. E. MacDonald, L. Eliasson, P. Rorsman, *J. Cell Sci.* **2005**, *118*, 5911.
[27] Y. Fujiki, A. L. Hubbard, S. Fowler, P. B. Lazarow, *J. Cell Biol.* **1982**, *93*, 97.
[28] B. W. Sutherland, J. Toews, J. Kast, *J. Mass Spectrom.* **2008**, *43*, 699.
[29] M. Dreger, *Mass Spectrom. Rev.* **2003**, *22*, 27.
[30] J. E. Celis, P. Gromov, *Curr. Opin. Biotechnol.* **1999**, *10*, 16.
[31] J. P. Whitelegge, S. M. Gomez, K. F. Faull, *Adv. Protein Chem.* **2003**, *65*, 271.
[32] D. Josic, J. G. Clifton, *Proteomics* **2007**, *7*, 3010.
[33] C. Pasquali, I. Fialka, L. A. Huber, *J. Chromatogr. B* **1999**, *722*, 89.
[34] J. R. Yates III, A. Gilchrist, K. E. Howell, J. J. M. Bergeron, *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 702.
[35] L. J. Foster, C. L. de Hoog, Y. L. Zhang, Y. Zhang, X. H. Xie, V. K. Mootha, M. Mann, *Cell* **2006**, *125*, 187.
[36] R. Cao, X. W. Li, Z. Liu, X. Peng, W. J. Hu, X. C. Wang, P. Chen, J. Y. Xie, S. P. Liang, *J. Proteome Res.* **2006**, *5*, 634.
[37] A. M. Rahbar, C. Fenselau, *J. Proteome Res.* **2004**, *3*, 1267.
[38] Y. A. Senis, M. G. Tomlinson, A. Garcia, S. Dumon, V. L. Heath, J. Herbert, S. P. Cobbold, J. C. Spalton, S. Ayman, R. Antrobus, N. Zitzmann, R. Bicknell, J. Frampton, K. S. Authi, A. Martin, M. J. O. Wakelam, *Mol. Cell. Proteomics* **2007**, *6*, 548.
[39] B. K. Shin, H. Wang, A. M. Yim, F. Le Naour, F. Brichory, J. H. Jang, R. Zhao, E. Puravs, J. Tra, C. W. Michael, D. E. Misek, S. M. Hanash, *J. Biol. Chem.* **2003**, *278*, 7607.
[40] L. Zhang, X. Wang, X. Peng, Y. Wei, R. Cao, Z. Liu, J. Xiong, X. Ying, P. Chen, S. Liang, *J. Proteome Res.* **2007**, *6*, 34.
[41] Y. X. Zhao, W. Zhang, Y. J. Kho, Y. M. Zhao, *Anal. Chem.* **2004**, *76*, 1817.
[42] E. L. Lawson, J. G. Clifton, F. Huang, X. Li, D. C. Hixson, D. Josic, *Electrophoresis* **2006**, *27*, 2747.
[43] T. Stasyk, L. A. Huber, *Proteomics* **2004**, *4*, 3704.
[44] D. Josic, M. K. Brown, F. Huang, H. Callanan, M. Rucevic, A. Nicoletti, J. Clifton, D. C. Hixson, *Electrophoresis* **2005**, *26*, 2809.
[45] B. J. Hoerl, R. E. Scott, *Virchows Arch. B* **1978**, *27*, 335.

  