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Investigation of Protein Patterns in Mammalian Cells and Culture Supernatants by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

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The direct protein profiling of mammalian cells and bacteria has a growing influence in biotechnology as a high information bearing method for characterization of cells and cell states. Monitoring of proteins excreted in culture media not only serves to produce data on product yield and quality but provides important information on cell viability and nutrient supply that forms the basis for future process and expression optimization. Fast and simple MALDI mass spectrometry approaches were developed to efficiently characterize such complex biological systems. Several mammalian cell lines including CHO DXB11, CHOSSF3, and hybridomas were investigated; the lysis process, the sample pretreatment, and the matrix preparation were optimized for MALDI conditions. Initial experiments to observe the success of protein translation in gene expression experiments were performed. Using MALDI-compatible detergents, it was possible to extend the mass range detectable by MALDI mass spectrometry from the current range of 16 000 to 75 000 Da. In this mass range, the data are complementary (offering a better mass accuracy) to those obtained by SDS−PAGE electrophoresis experiments. These new methods were used to monitor a large-scale cultivation of hybridoma cells expressing an antibody of the IgG type. The increase in whole antibody and antibody light-chain protein, 8650 Da, and the decrease of insulin were followed during the monitoring period. Quantitative measurements of the IgG level during the cultivation compared favorably with those obtained by affinity HPLC.

In the past decade, MALDI mass spectrometry has established its role as an important analytical tool for protein chemistry as well as for molecular biology.[1,2] Some key advantages of MALDI mass spectrometry render this technology especially suitable for the characterization of biologically relevant molecules in complex media. The absence of fragmentation, high sensitivity, and easy sample preparation are the main advantages of this analytical tool. This has led to the introduction of MALDI for the analysis of rather complex systems where classical approaches have proven to be complicated and tedious, including the rapid identification of pathogenic and nonpathogenic bacteria[3−6] and the determination of gluten in food.[7] The potential applicability of MALDI mass spectrometry for profiling biological cell reactions is investigated in this paper.

Animal cell cultivation is becoming increasingly important for the production of pharmaceutically active proteins ranging from vaccines (measles, polio, hepatitis A) to proteins with therapeutic effects (TPA, TGF-β, α-interferon, monoclonal antibodies).[8] Exactly defined and controlled cultivation procedures for cells, tissues, and organs are required to obtain and maintain a stable and high-quality production.

MALDI mass spectrometry can easily detect and monitor subtle changes in the pattern of intracellular molecules and in the compound profile in cell cultivation liquids. Prerequisites for such applications are a careful selection of matrix compounds and solvents and especially a high reproducibility in the matrix embedding and crystallization process.[9] Compared to prokaryotic cells such as *Escherichia coli*, mammalian cells exhibit a far higher complexity. Composed of a wide variety of peptides, proteins, glycoproteins, oligonucleotides, lipids, and sugars, these cells offer a challenge to any kind of mass spectrometry, especially since larger amounts of inorganic salt and detergents are often present in the sample due to separation and stabilization requirements. This holds equally true for cell cultivation liquids. Up to now, MALDI mass spectrometry could only detect small proteins in cell lysates up to a molecular mass of 20 000 Da—whereas SDS−

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page electropherograms revealed the presence of proteins with a molecular mass range up to several hundred kilodaltons. Some of the reasons for this discrepancy are discussed in this paper.

Optimizing productivity of mammalian cells relies on developing suitable methods for screening and selection of high producing cultures. Additionally, understanding and control of regulation and expression mechanisms are of key importance. This means in the case of hybridoma cells fused to produce monoclonal antibodies, investigated here, the regulation of antibody expression, assembly, and secretion have to be understood.[10,11]

For mammalian cell lysis, different techniques have been developed over the years. A disadvantage of most of these protocols is the biased selection of certain protein classes occurring during the course of the lysate preparation. Almost all the protocols include purifications that yield a specific group of compounds for analysis rather than measurement of the cell in its entirety. To find acceptance as a routine analytical method for monitoring cell cultivation, it is necessary to yield quantitative or at least semiquantitative data from cell culture medium. In this paper, we will show that the concentration of insulin, an important component of the medium for certain cell lines (it stimulates protein and lipid synthesis), can also be measured using MALDI mass spectrometry.[13−15] In the past, MALDI has already shown its potential to be a semiquantitative tool in other applications.[12] Experience has shown, however, that special procedures are necessary to obtain results of similar quality in complex biological systems. In this paper, approaches yielding optimal quantitative results are outlined.

EXPERIMENTAL SECTION

Cell Cultures. The CHO DXB11, a Chinese hamster ovary (CHO) cell line negative for dihydrofolate reductase (dhfr-), is the parental cell line of CHOSSF3 and CHOSSF6.[16] The suspended serum-free (SSF) cell lines were grown in FMX-8,[9] serum, and protein-free medium specially designed for these cell lines. This FMX-8 medium (F. Messi AG, Zürich) is described in detail by Zang et al.[17] It is composed of high concentrations of low-molecular-weight molecules, including vitamins and amino acids as well as salts and sugar. For the measurements of the intracellular and extracellular production of the IgG light chain, a transfected CHOSSF3 cell line, CHOSSF3.pMOZ.LC, was used. The description of this cell line and the transfection can be found in the paper of Zang and co-workers.[17] This cell line grows in trp-selection medium (FMX-8 without tryptophan, supplemented with 300 μM indole). The CHO DXB11 cell line grows adherent in FMX-8 supplemented with 5% fetal calf serum (Gibco). A549 is a human lung carcinoma cell line (ATCC-CRL-7909) and was cultivated in DMEM (Gibco Life Technologies Co., Gaithersburg, MD) supplemented with 10% fetal calf serum. Hybridoma cells are fusion cells of an IgG-producing B-cell and a myeloma cell. They were cultivated in PFHM (Gibco) supplemented with 5 mg/L insulin and 5 mg/L transferrin. All cultures were cultivated in T-flasks and were incubated in 5% CO_2 and a 37 °C incubator. For the measurement of glucose concentration, the glucose assay kit of Boeringer Mannheim was used.

Cell Lysates. Approximately (2−5) × 10^5 cells were centrifuged 5 min at 1000*g* and the cell pellet was washed three times with cold phosphate-buffered saline (PBS). The wet pellet was resuspended in 100 μL of lysis buffer (5 mmol/L triethanolamine, 0.2 mmol/L EDTA, pH 7.4) and incubated for 30 min on ice. Then the cell suspensions were subjected to three freeze (liquid N_2) and thaw cycles. The cell lysates were directly measured or stored at −80 °C. No deterioration was detected upon storage. Prior to measurement, cell debris was removed by centrifugation. If the cells grew adherent, the medium was removed and the cells were rinsed with PBS. Using a cell scraper, the cells were scraped off the bottom into the lysis buffer.

Detergents. To expand the mass range of the MALDI measurement, usually Triton X100 was added to 0.1% in 10 mL of the debris-free cell lysate solution. If removal of the detergent was necessary, 20 μL of Triton X100 adsorbing beads (Calbisorb, Calbiochem-Novabiochem Corp., La Lolla, CA) was added. After incubation for 5 min at room temperature, the beads were removed by a gentle centrifugation step. The supernatant contained the detergent-free protein solution.

Removal of Lipids and Lipoproteins from the Cell Lysates. A 10-mg sample of PHM-L Liposorb absorbent (Calbiochem, La Jolla, CA) was equilibrated in 120 μL of 20 mM HEPES, pH 7.4, for 30 min. Cell lysates were mixed 1:1 with the Liposorb solution and incubated overnight at 4 °C. Prior to the measurements, cell debris and the adsorbent were removed by centrifugation. The samples were further treated as described under MALDI mass spectrometry sample preparation.

Fractionation of the Cell Lysate. Cell lysate fractionation by molecular weight was performed by ultrafiltration filters with a molecular weight cutoff of 3000 and of 10 000 from Amicon (Beverly, MA) as well as Millipore (Bedford, MA). The filters were used as recommended by the producer. Cell lysates were filtered, and the concentrated sample on the filter as well as the filtrate were measured by MALDI mass spectrometry.

For the fractionation of the cell lysate according to charge, anionic and cationic exchange units (Sartobind, S5 and Q5, Millipore) were used in a FPLC system (Pharmacia, Biotechnology, Uppsala, Sweden). The units were loaded with 2 mL of cell lysate (2 × 10^6 cells) and eluted with buffer A (10 mM HEPES, pH 7.2) and buffer B (10 mM HEPES, 500 mM NaCl, pH 7.2) in a gradient from 0 to 100% buffer B in 30 min and a flow rate of 1 mL/min. The flow-through and the subsequent elution fractions were characterized by MALDI mass spectrometry. In parallel, these samples were also analyzed by SDS−PAGE and DNA gel electrophoresis using standard methods (Data not shown).

Culture Supernatant. Culture samples were centrifuged for 5 min at 1000*g*, and the clear supernatant was collected. For these culture supernatants, further sample pretreatment was necessary before MALDI measurements were done otherwise the high load of phosphate buffer, amino acids, and glucose interfered with the

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measurement. Drop dialysis on a floating membrane was done to desalt the samples in the low-microliter range.[18,19] For this purpose, a cellulose mixed ester (MF membrane, Millipore) was used as the floating membrane on a buffer reservoir. A 150 mM diammonium hydrogen citrate solution was used to effectively remove alkali ions and 0.1% Triton X100 without protein loss by adsorption. The dialysis time was always kept at 40 min. It should be noted that the dialysis time and the buffer concentration are very critical for quantitative measurements. For typical measurements, four 20-μL droplets were placed on the 25-mm-diameter membrane. After dialysis, the complete sample droplets were collected using a micropipet.

MALDI Mass Spectrometry. All MALDI spectra were obtained using a LDI-1700 instrument (Linear Scientific Inc., Reno, NV) with a pulsed nitrogen laser (337 nm). The vacuum inside the 1.7-m flight tube was always kept between 10^{-7} and 10^{-6} Torr. For deflecting the matrix and other small ion signals, a mass filter was set at 3000 Da. All spectra were obtained by the accumulation of 50 shots in the positive mode. For the MALDI-MS measurements, 3 μL of a given sample was mixed with 3 μL of ferulic acid in a 2-propanol/water solvent (1:1).[20] It was found in previous tests that this matrix together with 2-propanol has several advantages in comparison with other matrix systems. A much higher analyte-to-matrix ratio could be tolerated and higher peak intensities in the upper mass range could be achieved, while suppression effects were reduced. It should be noted that the sample/matrix mixture was rapidly dried by vacuum using the crystallization accessory (HP G2024A; Hewlett-Packard, Palo Alto, CA) resulting in a regularly distributed fine microcrystalline layer. This procedure was necessary for obtaining reproducible quantitative data. To allow comparisons of peak intensities in different spectra, 30 mg/mL phospholipase D from white cabbage (Fluka, Buchs, Switzerland) was added before the dialysis process. For quantitation, the mass spectra were normalized using features of the MALDI software.[21] If necessary, a HEPES buffer was used for dilution because of its good compatibility with the matrix.[22]

SDS−Gel Electrophoresis. SDS−gel electrophoresis was performed using the PhastSystem (Pharmacia LKB Biotechnology, Uppsala, Sweden). The gels were stained with coomassie blue using the development unit of the same instrument. All measurements were carried out with 1.4 g of SDS/g of protein and heating at 100 °C for 5 min. A gel 43 × 60 mm in size (homogeneous 12.5%) with a usable mass range between 15 and 250 kDa was used for Figure 4. A 4-μL sample of lysate solution and 1 μL of standard were applied to the sample applicators. For calibration, the HMW-SDS kit with a mass range from 53 000 to 212 000 Da and the LMW electrophoresis kit with a mass range between 14 400 and 94 000 Da were used. The samples were concentrated by centrifugation at 14000*g* for 2 min in a 10 000 Da cutoff centrifuge concentrator (Microcon) from Amicon Inc.

RESULTS AND DISCUSSION

MALDI-MS technology has reached an extremely high level of performance, yielding results that quite often rival those obtained by multisector instruments. However, one of the basic physical parameters in the MALDI process is the correct embedding of the sample into the matrix. Especially when dealing with complex biological mixtures, all the unavoidable additives such as buffers, detergent, and cell debris have to be accommodated. In an effort to improve the quality of MALDI spectra, we carried out the following steps in order to optimize the preparation of cell lysates and culture supernatants. To overcome the limited mass range of cell lysate samples, the effects of lipids, DNA, detergent, and fractionation have been studied. In addition to information obtained from cell lysates, culture supernatants contain additonal important information that can be obtained by monitoring key protein concentrations using MALDI-MS.

Evaluation of Cell Lysing Methods. At the beginning of our MALDI mass spectrometry investigation of cell lysates, we used standard matrixes such as sinapinic acid in acetonitrile, known for many years for the characterization of proteins. The results obtained were poor: small peak intensities and a rather limited mass range. Only a few peaks below 6000 Da were visible and none above this limit. Our first step was cell lysis optimization. It was obvious that no further molecules should be added due to the already high complexity of the sample. To avoid any interference with the MALDI measurement, no inhibitors for proteases, detergents, or high salt concentrations were used for cell lysis. To limit the effect of proteases, samples were stored on ice during the measurements or at −80 °C over longer periods. It was found that the matrix itself could reduce the enzyme activity when added during cell lysis. A combination of buffer containing triethanolamine and several freeze−thaw cycles for cell lysis resulted in the best peak intensities. This combination dissolved mostly cytoplasmic proteins. The efficiency of this lysis procedure was checked by SDS−PAGE electrophoresis. Results obtained were promising; however, the MALDI data still were not acceptable. Most likely the process of crystallization that should yield a uniform microcrystalline layer was obstructed with byproducts. For the next step, matrixes that should be capable of producing high peak intensities were evaluated.[20] It was found that ferulic acid, already known for many years, gave quite promising results for synthetic protein mixtures for the high-molecular-mass range (>150 kDa). However, more significant improvements were achieved with another solvent, used for the preparation of the matrix.[21] With ferulic acid in a 2-propanol/water mixture, high peak intensities and an extended mass range were obtained. We found that this system even tolerated high concentration levels of proteins and salts as well as certain detergents (see below). High peak intensities in the upper mass range without discrimination of proteins in the low-molecular-mass range were reached. Direct measurements of cell pellets after a freeze−thaw cycle resulted in a useful mass spectra.

Cell Lysates of Different Cell Lines. To prove the applicability of the optimized procedures, different mammalian cell lines were tested: CHOSSF6 and its parental cell line CHO DXB11; A549, a human lung carcinoma cell line and a hybridoma cell line, derived by fusion of a B-cell and a myeloma cell producing IgG (Figure 1). Surprisingly, given the presumably identical basic

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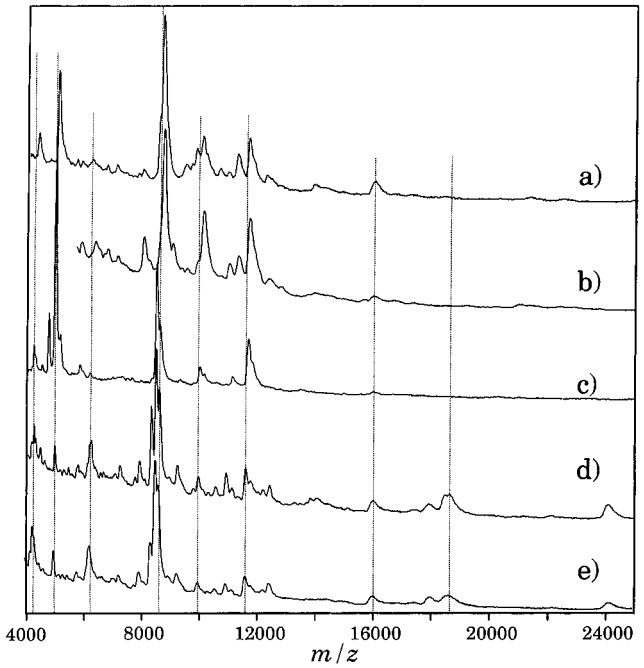

Figure 1. Positive ion spectra of lysates of different mammalian cell strains. The spectra show the intracellular protein patterns of CHO DXB11 (a), CHOSSF6 (b), A549 (c), and two different batch cultivation of hybridoma cells for reproducibility (d, e). The vertical lines indicate some main peaks for a better comparison between the different cell strains.

metabolic processes in mammalian cell lines, the profiles of the different strains vary significantly. As expected, the spectra of the CHOSSF6 and of CHO DXB11 were closely related, reflecting the close genetic relationship (CHOSSF6 is derived from CHO DXB11 by mutation using ethyl methanesulfonate as a mutagen[18]). To show the reproducibility of the method, spectra of two different batches of hybridoma cells cultivated under identical conditions are shown in Figure 1d and e. The samples were taken in the mid-log phase of the culture. The peak at 24 kDa represents the monoclonal antibody light chain produced by the hybridoma cells. Only small differences in the spectra were obtained, mirroring small differences between cultivations of the same cell lines, whereas the main pattern remained constant, showing exactly the same predominant peaks.

With this method, no proteins with molecular masses larger than 24 000 Da could be detected. SDS−PAGE, however, indicated the presence of proteins up to 116 000 Da in the very same sample. Due to the high mass resolution of MALDI mass spectrometers, even with the limited mass range this method proved to be suitable for screening purposes.

Effect of Lipids in Cell Lysates. With the combined method of freeze−thaw cycles in liquid N_2 and use of the lysis buffer for cell lysis, water-soluble proteins were efficiently released. The presence of lipids and lipoproteins, however, cannot be completely excluded. Lipids and lipoproteins are known to have an influence on the measurement of complex biological samples with MALDI-MS. With the help of a lipid absorbent (Liposorb), the effect of lipids and lipoproteins was investigated. It was found that further improvements for the MALDI spectra could be reached when all lipids were removed from the cell lysate solution. Profiles of native CHOSSF3 cell lysates compared to the identical sample where

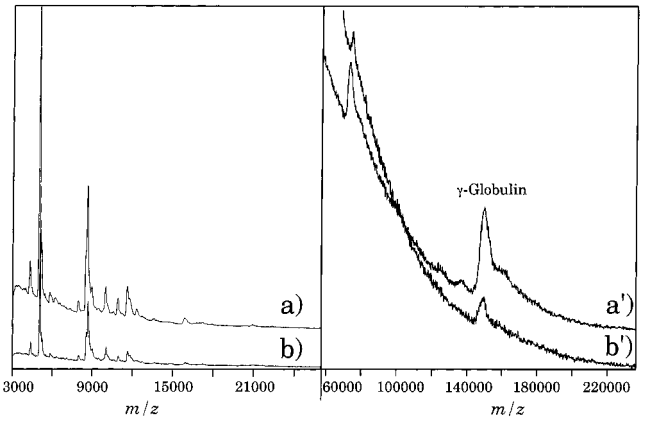

Figure 2. Effect of lipids on the signal intensity from cleared cell lysate solutions spiked with 100 μg/mL γ-globulin. Positive ion mass spectra with different mass areas after desorbing with 20 μL of lipid-adsorbing beads (a and a′) and without adsorbing beads (b and b′).

lipids were removed by using lipid-adsorbing beads showed a 3-fold improvement in the signals upon removal of the lipid contaminants (Figure 2). Spiking the cell lysate solution with γ-globulin (147 kDa) showed that the improved sensitivity also could occur for proteins with higher molecular weights (Figure 2a′ and b′).

Fractionation of Cell Lysates. The high concentration of low- and mid-molecular-weight compounds with a large heterogeneity might impact the embedding process by hindering the crystallization process. Fractionation of the cell lysates using membranes, however, resulted in deteriorated spectra, most likely due to high absorption losses. Fractionation on cation-exchange resins at pH 7.2 and 5.0 failed due to extremely low binding/retention. The anionic exchange unit did bind proteins, resulting in two fractions, the unbound proteins in the flow-through and the bound proteins in the eluate just after starting with the salt gradient. The measurements with MALDI-MS, however, did not reveal new peak signals or an increase in peak resolution. As the anion-exchange process removes DNA very efficiently, a possible impact of DNA on spectral performance should be detectable. Introduction of proteins for changing the crystallization mechanisms of the MALDI matrix could deteriorate the spectrum. No significance change, however, could be observed. In summary, none of the possible advantages of the fractionation processes outbalanced the losses due to adsorption.

Effect of Detergents in Cell Lysates. With the improvements described above, there was still a detectable mass limit at about 25 000 Da when original lysates were used. Spectra obtained after spiking with proteins such as γ-globulin (Figure 2a′ and b′) indicated that the determination of ions with higher molecular weight would be possible. A total protein determination of the cell lysates (Bradford) showed a much higher protein concentration than the MALDI spectra. The use of detergents (0.1−0.6% Triton X100) as matrix additives was tested to improve resolution of higher molecular weight proteins. When 0.1% Triton X100 was added to the cell lysates of different cell lines, the mass range could be extended to 75 000 Da (Figure 3). These data have now some similarities to these obtained by SDS−PAGE (Figure 4b−d); however, they are still not identical. In the range up to 75 000 Da, both MALDI and SDS−PAGE show a similar number of peaks. In comparison with the protein peak pattern observed

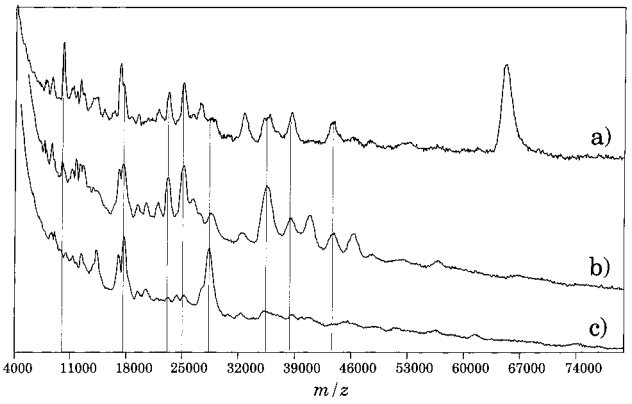

Figure 3. Positive ion spectra of different cell lysates after 0.1% Triton X100 was added to the cleared solution: (a) CHO DXB11, (b) CHOSSF3.pMOZ.LC, and (c) hybridoma. The vertical lines indicate some main peaks for a better comparison between the different cell strains.

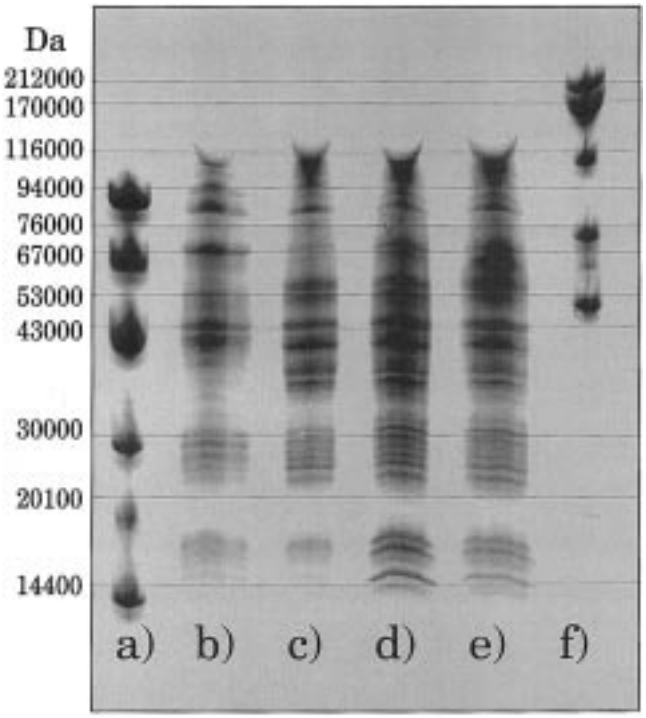

Figure 4. SDS−PAGE electrophoresis of different cell lysates: (a, e) markers, (b) hybridoma, (c) CHOSSF3.pMOZ.LC, and (d) CHO DXB11.

without the detergent (Figure 1a), the main peaks disappeared after the addition of the detergent. Since cell lysates contain quite a variety of hydrophilic as well as hydrophobic proteins, we assume that during the crystallization process the hydrophilic proteins were embedded more uniformly into the matrix whereas the hydrophobic moiety was barely integrated and thus not visible in the spectrum. Also, Rosinke et al. have shown that the embedding of membrane proteins in a matrix deteriorates the signal.[23] When detergents such as Triton X100 or Tween 80, which are quite compatible with the MALDI process itself,[20] are used, such effects can be reversed. When these detergents (0.1−0.6%) are added to the clear sample (lysate) solution, only the

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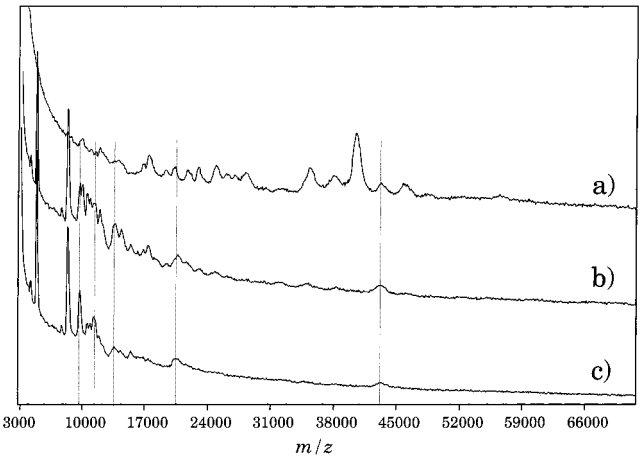

Figure 5. Effect of detergent addition to a cleared solution of CHOSSF3 cell lysate: (a) positive ion spectrum after adding of 0.61% Triton X100, (b) mass spectrum after removing the detergent by adsorbing beads, and (c) comparison with pure cell lysate solution. The vertical lines indicate some main proteins for a better comparison between the spectra.

hydrophobic proteins become visible in the mass spectra, whereas the hydrophilic proteins are suppressed. An additional experiment showed the reversibility of these effects (Figure 5). First, clear cell lysate was measured by MALDI without detergent and then 0.1% Triton X100 was added, resulting in a profile where high-molecular-weight compounds became visible (Figure 5a). Finally, the added detergent was removed using Calbiosorb beads; the profile obtained then was quite comparable to the original spectrum (Figure 5b and c). Sample loss due to precipitation was not observed in any of these experiments; when these new data are compared with SDS−PAGE it becomes clear that the MALDI spectrum with Triton X100 is now complementary with the gel results.

The observed effects after adding and removing detergent can be explained by the hydrophilic or hydrophobic character of cell proteins. A key step in matrix-assisted laser desorption and ionization is the cocrystallization process of matrix and sample molecules. The system must crystallize in such a way that the sample molecule can be embedded into the matrix lattice in an evenly distributed manner. It is well-known that different crystallization methods and also different matrixes can produce different spectra of a given protein mixture. Figure 3 indicates that after adding a small amount of detergent all hydrophobic proteins are declustered and embedded as single molecules inside the matrix during the crystallization process. However, when no detergent is added, these hydrophobic proteins will agglomerate to larger units, which will not be detectable by standard MALDI. The hydrophobic/hydrophilic effect also explains some of the effects shown in Figure 2a and b. Here the CHOSSF3 cell lysate was spiked by 100 μg/mL γ-globulin (6.7×10^{-7} mol/L, ≅1.3 pmol on the probe tip). This more hydrophilic molecule of 147 kDa was easily detected in this complex cell lysate solution. Additional experiments, however, are necessary to fully explain all the effects observed.

Application: Monitoring of Gene Expression. Data obtained by analysis of the intracellular protein pattern and the proteins secreted in the culture supernatant allow monitoring of the production of proteins after genetic manipulation of the cell

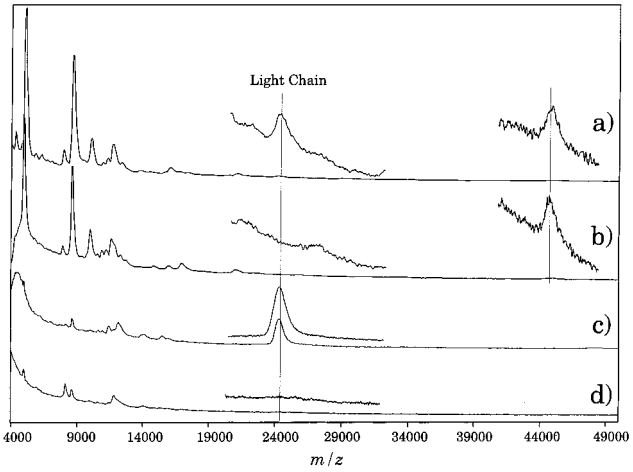

Figure 6. Positive ion spectra of cell lysates and culture supernatant of CHO SSF3/pMOZ IgG LC cells producing light chain: (a) SSF3/pMOZ IgG LC cell lysate, (b) SSF3 cell lysate, (c) SSF3/pMOZ IgG LC culture supernatant, and (d) SSF3 culture supernatant. The vertical lines indicate the mass of light chain for a better comparison between the different spectra.

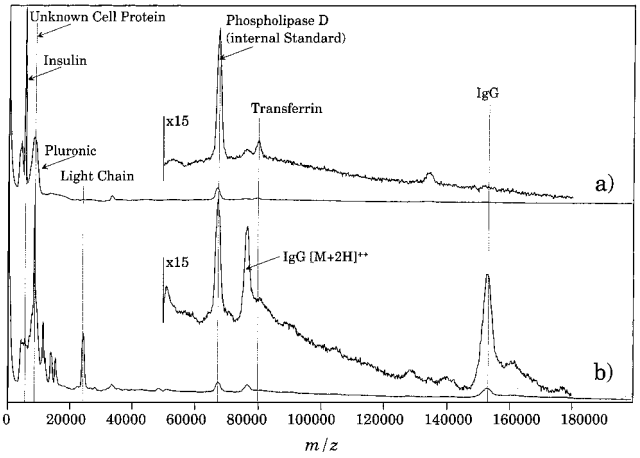

Figure 7. Positive ion spectra of the culture supernatant from hybridoma cells that produce IgG at the beginning (a) and the end (b) of a batch cultivation. Phospholipase D was used as an internal standard for semiquatitative determinations. The vertical lines indicate some main proteins for a better comparison between the two spectra.

and thus the success of a transfection. To check the applicability of MALDI-MS for the testing of producer clones, the supernatant and the cell lysates of a CHOSSF3 cell line producing IgG light chain (CHOSSF3.pMOZ.LC) and the mother cell line CHOSSF3 were measured (Figure 6). The IgG light-chain signal (24 kDa) could be detected in the cell lysate as well as in the culture supernatant after protein expression. Figure 6a shows the profile of the system after the induction of IgG light-chain expression. Figure 6b as a reference shows the profile of the nonproducing cell line. The peak close to 44 kDa is a cell protein detectable in the cell lysates of both cell lines. This peak can be used as an internal standard for comparison of the intensities. Investigation of culture supernatant after cell cultivation also shows the presence of the light-chain protein as a new peak in the spectrum (Figure 6c). Figure 6d shows the result of the culture media of the nonproducing cell line. Comparison of spectra a and c shows a higher concentration of the light chain in the cultivation medium. This indicates that the light chain formed by the cell is not accumulated in high amounts within the cell but mainly excreted in the media.

The small amount of cells or culture supernatant needed as well as the quick assay qualifies MALDI-MS as a good alternative for the ELISA measurement, which is normally applied for expression monitoring, especially as no specific antibodies are required. An additional advantage of the measurement is the possibility to detect additional intracellular and extracellular parameters; this additional information can be used to identify the reason for the loss of protein production that might be based on genetic instability or problems associated with the assembly or secretion of the protein.

Information Contained in the Culture Supernatant. The experiments related to gene expression indicated that the culture supernatant contained significant information about the cells themselves, as well as the condition of the culture medium. During the cultivation process, the cell will actively or passively release compounds into the culture medium and metabolize nutrients. However, the measurement of crude culture supernatant did not give acceptable spectra showing any significant information. Therefore, a sample pretreatment step was introduced. After drop dialysis on a floating membrane, the MALDI-MS spectra revealed more information. Figure 7 shows two MALDI-MS spectra of culture supernatant at the start and the end of a batch cultivation of hybridoma cells, cultured in serum-free medium supplemented only with insulin and transferin. The spectrum of fresh culture medium (Figure 7a) showed signals for insulin at 5803 Da on top of the broad peak of Pluronic F68 (detergent), with the top at 8650 Da, and for transferrin at 79 950 Da. This spectrum is compared with that obtained at the end of batch cultivation (Figure 7b). Phospholipase D was added as internal standard for quantitation purposes. We observe an increase in the signals of the main products, intact IgG, and the respective light chain and also a decrease of the insulin signal and the occurrence of new peaks. The shoulder of the antibody peak relates to a Pluronic adduct. With control experiments, it was found that Pluronic interacts noncovalently with IgG, but not with other proteins, such as phospholipase D. The noncovalent binding of Pluronic to IgG may be a reason for interference with traditional immunological measurement methods such as ELISA. The new signals in the culture supernatant might reflect the secretion of peptides or small proteins by living cells as well as the passive release from dead cells after breakdown of the cytoplasmic membrane. Also, degradation of excreted proteins by proteases may cause small peptide fragments in the supernatant. However, comparison of the peak pattern of hybridoma cell lysate with the culture supernatant at the end of the batch culture showed that peaks of the cell lysate appeared in the culture supernatant as well. This points to a direct link between proteins produced in the cell and their appearance in the cultivation medium.

Thus, the measurement of the culture supernatant with MALDI mass spectrometry may give information on the growth and production of animal cells in culture and could be used as a tool for monitoring cell cultures over time.

To support this statement, hybridoma cells were cultured in a 20-L batch bioreactor under standard conditions. Samples of the

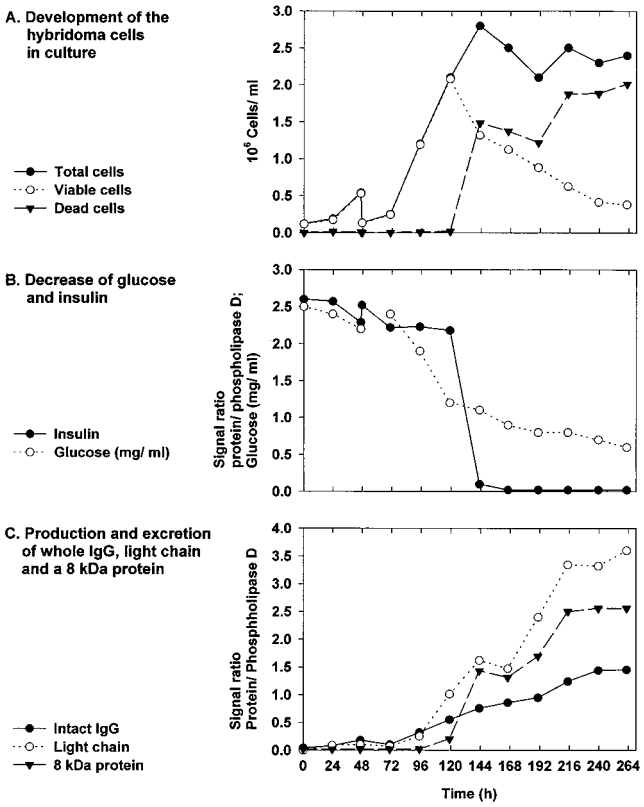

Figure 8. Batch cultivation of hybridoma cells in a 20-L aerated stirred tank bioreactor under standard conditions (37 °C) in PHM-medium supplemented with 5 mg/L insulin and 5 mg/L transferrin. (a) Proliferation of the hybridoma cells, measured by the trypan blue dye exclusion method. (b) Decrease of the glucose and insulin concentration in the medium. Insulin was measured using MALDI-MS. Signal of insulin relative to the phospholipase D signal in the same spectrum. Glucose was measured as described in the Experimental Section. (c) Production and secretion of whole IgG, light chain, and a 8-kDa protein as measured with MALDI under the same conditions as insulin. MALDI data are the means of three measurements. At $t = 47.5$ h the culture was diluted (feeding) 1:4 with fresh medium. The data points at 48 h were calculated using the measurement of $t = 47$ h.

culture supernatant were regularly taken for measurement with MALDI-MS, following in particular the fate of insulin, a cell protein at 8 kDa, and the production of light chain and intact antibody. All mass spectra were normalized using the peak intensity of the phospholipase D as internal standard. In parallel to the MALDI-MS measurement, cell numbers (dead and alive) and the glucose concentration were measured. Figure 8 gives an overview of the data obtained. After 48 h of cultivation, the culture was diluted 1:10 with fresh medium (feeding). At 120 h, the viable cells reached their maximum concentration of 2×10^6 cells/mL, while at later times, the viable cell number decreased steadily (Figure 8). Between 120 and 144 h, a rapid decrease in insulin concentration to a nearly undetectable level was observed as measured with MALDI-MS (Figure 8B). A direct correlation between the decrease of the glucose and the insulin concentration could not be observed. The glucose concentration decreased at a high rate during the log phase (between 72 and 120 h) but decreased more slowly at later times. However, a possible relation between the glucose consumption rate and the insulin concentration cannot be ruled out. Control experiments of cell-free spent medium

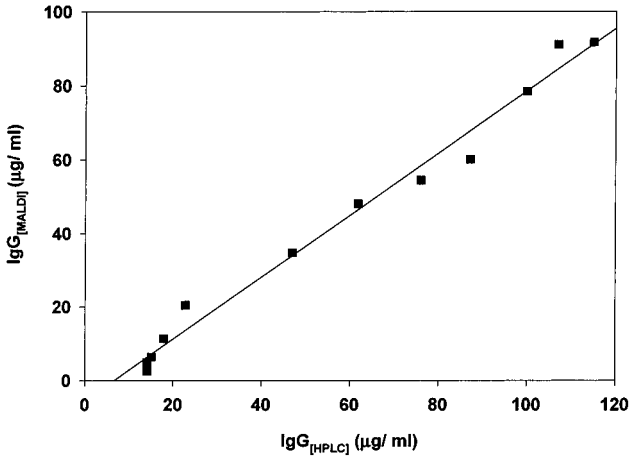

Figure 9. Comparison of the IgG concentration in the culture supernatant measured by HPLC and MALDI-MS ($R^2 = 0.985$).

spiked with insulin and incubated at 37 °C did not reveal a dramatic decrease in the insulin content (data not shown). Thus we conclude that insulin was primarily consumed or bound by the cells. The rapid decrease in insulin may point to a change in metabolism of the cells in culture concomitant with the change in growth phase, from the log phase to stationary, decline phase.

The production and secretion of intact IgG and light chain were also followed over time during the batch cultivation using MALDI-MS (Figure 8C). It was found that the amount of IgG light chain secreted in comparison to intact IgG was 1:1 up to 96 h and then the ratio shifted to around 3:1 at the end of cultivation. This effect may be due to the released of light chain by dead or dying cells. As already mentioned, animal cells produce light and heavy chains separately which then are assembled intracellularly into whole IgG, which is secreted; the light chain is overproduced and the excess is normally broken down intracellularly. The shift in the ratio of light chain to intact IgG may be due to a passive release of light chain by dead or dying cells after disruption of the cell membrane. But changes in the regulation of the entire process, including, assembly and secretion, toward the end of the batch cultivation due to limitation of essential medium components or to toxic effects of waste products, cannot be ruled out.

For a production cell batch, it is always important to determined the exact concentration of expressed proteins. Therefore, a calibration curve was established for immunoglobulin. To calibrate the MALDI instrument, stock solutions of γ-globulin in different concentrations were mixed with the same concentration of phospholipase D. The peak ratio between the γ-globulin and the phospholipase D were calculated for each mass spectrum. To create realistic conditions, fresh culture media was used as solvent. Also the sample preparation step was highly optimized to be quantitative. The standard deviation of the regression line was determined to have a good reliability factor ($R^2 = 0.993$) including the dialysis step. The curve was found to be linear up to a concentration of 400 μg/mL γ-globulin. With this calibration curve, the IgG concentration was calculated for each sample. The comparison of the monitored IgG concentration as measured with MALDI-MS and with HPLC using affinity chromatography delivered compatible curves (Figure 9). The small differences between the MALDI-MS and HPLC results may be due to impurities in the γ-globulin used as standard.

During the monitoring of the batch cultivation, an unknown protein with the mass of 8560 Da increased in concentration during the culture development (Figure 8C). The trend of this protein followed that observed for the appearance of dead cells (Figure 8A). The same molecular mass peak could also be detected in cell lysates of the hybridoma cells. It would be appealing to follow upon the suspected parallelism of cell viability and appearance of the 8560-Da protein to test its use as an early indicator of cell death.

CONCLUSIONS

The present investigations have demonstrated the applicability of MALDI mass spectrometry as an analytical tool for biotechnology. We showed that complex biological systems can be characterized with few sample pretreatment steps if the factors that impact spectral quality are understood and controlled. Under controlled conditions, quantitative results suitable for monitoring biological processes can be obtained. Careful selection of measuring conditions can expand the visible mass range up to 75 000 Da for the determinations of proteins and glycoproteins in cell systems, making the results complementary with standard SDS−PAGE electrophoresis. The use of detergents in cell lysates might be also an useful for the investigation of particular proteins, which do not give any signal under standard MALDI-MS conditions. Key factors that negatively impact the spectral quality are identified; means to remedy such situations are introduced. MALDI-MS can be successfully used for profiling and discriminating related cell lines, quantitative monitoring of gene expression, and monitoring complex cell cultivations. Comparison of MALDI-MS results with established alternatives shows the high precision, high speed, and high information context of MALDI mass spectrometry.

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