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Protein quantification by the SELDI-TOF-MS–based ProteinChip® System

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Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) represents the successful combination of retentate chromatography and mass spectrometry, and this technology is an integral part of Ciphergen's ProteinChip System, which was designed to answer biomedical questions by performing protein analyses on a single experimental platform. The quantification capability of the ProteinChip System is essential in all proteomic applications for which this technology is used. Here we describe methods and results for three short experiments mimicking realistic analytical challenges to provide practical examples of quantification.

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SELDI and MALDI

SELDI-TOF-MS can be considered as an extension of the matrix-assisted laser desorption/ionization (MALDI)-TOF-MS method. In both cases, proteins to be analyzed are cocrystallized with UV-absorbing compounds and vaporized by a pulsed-UV laser beam. Ionized proteins are then accelerated in an electric field, and the mass to charge ratios of the different protein ion species can be deduced from their velocity. The differences between SELDI and MALDI are in the construction of the sample targets, the design of the analyzer and the software tools used to interpret the acquired data.

In the SELDI method, protein solutions are applied to the spots of ProteinChip Arrays, which have been derivatized with planar chromatographic chemistries. The proteins actively interact with the chromatographic array surface, and become sequestered according to their surface interaction potential as well as separated from salts and other sample contaminants by subsequent on-spot washing with appropriate buffer solutions. Furthermore, protein interaction studies or enzymatic reactions may be carried out directly on-spot under physiological conditions. The chromatographic surfaces provide a very good support for the cocrystallization of matrix and target proteins, resulting in the formation of a homogenous layer on the spot, thereby delivering an ideal crystalline surface for the subsequent analysis.

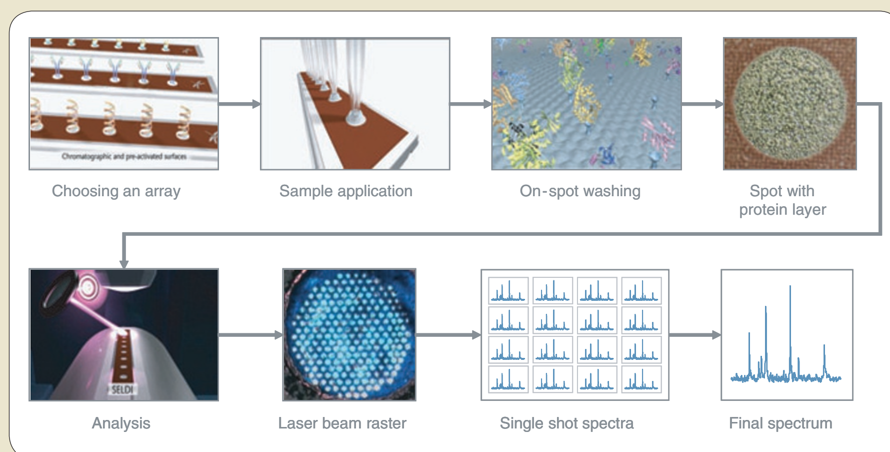

Figure 1 | The experimental steps**.** After choosing an array from a selection of chromatographic and preactivated ProteinChip Arrays, samples are applied and incubated on the spots. On-spot washing ensures efficient sample cleanup, and the spot surfaces allow the formation of a homogenous layer of cocrystallized proteins and matrix compounds. In the ProteinChip Reader, a laser beam is directed on the spot causing desorption and ionization of the proteins. A defined laser beam raster is used to selectively cover the entire spot surface and allows repeated reading of a single spot without using the same positions twice. Multiple spectra from a statistically meaningful area are then averaged in a final spectrum in which the mass-to-charge ratios of the ionized proteins are given and a good correlation between signal intensities and analyte concentration is achieved for the different peptides and proteins in the sample.

Sample preparation for SELDI experiments is quite different than the process for MALDI. For MALDI analysis, protein solutions are typically premixed with the matrix and dried on a passive surface. With the exception of flash washing with cold distilled water, on-target purification is not possible, and pre-target deposition sample cleanup procedures must be applied to reduce chemical noise and ion suppression. Also, on-target segregation of protein populations

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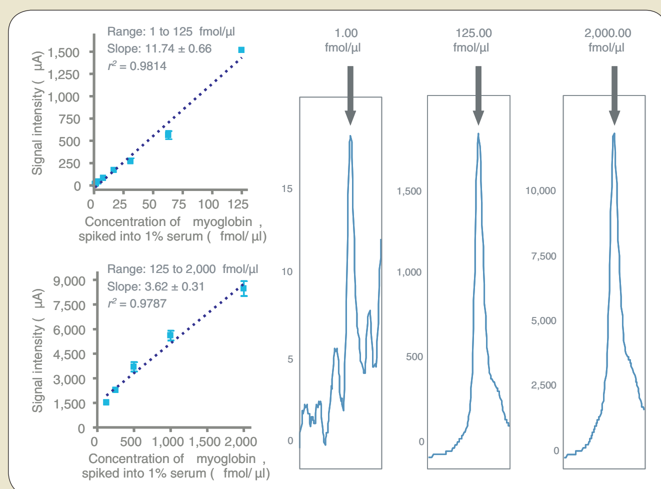

Figure 2 | Quantification of myoglobin in 1% serum on chromatographic arrays. As shown by the graphs on the left, the analysis revealed a dynamic range of 1.00 to 2,000.00 fmol/µl with very good linear correlation for the 1 to 125 fmol/µl and the 125 to 2,000 fmol/µl ranges. Data are mean ± s.e.m. from five replicates. The three panels on the right show representative signals for the experiment with myoglobin protein, demonstrating equally good signal quality over the entire concentration range.

is not practical because the surface has only weak and unpredictable interaction properties. For these reasons, prefractionation using a variety of microtechniques is often used. Taken together, these sample preparation requirements complicate the MALDI analysis, often resulting in sample loss as well as artifactual qualitative and quantitative variances.

The analyzers used for SELDI and MALDI were designed with different purposes in mind. The ProteinChip Reader is especially adapted to achieve high-sensitivity quantification and good reproducibility. The ion source and detector are constructed to support very efficient ion transmission and ion detection over a wide mass range. The precise positioning of the laser beam is controlled by software both in manual and automatic mode. The process is visualized in a user-friendly format by a pixel raster map to facilitate the multiple analyses of the same sample spot, and software tools allow normalization of the resulting spectra to their total ion current for internal quantitative calibration. These features assure high precision and reproducibility even when great numbers of complex biological samples need to be comparatively analyzed.

In contrast, MALDI devices are not designed for reliable quantitative precision over a wide mass range. They are a very good choice if high accuracy in the lower peptide range is needed without a requirement for high reproducibility of signal intensities. But if a good correlation between signal intensities and protein concentration is to be achieved over a wide mass and sample concentration range, the SELDI-TOF-MS–based ProteinChip Reader will always produce data with better reproducibility for hundreds of samples per day.

The quantification capability and reproducibility make the ProteinChip System a robust tool to address biomedical questions in the proteomics field[1]. Not only is this true for biomarker discovery and assay development[2] but also for protein interaction studies[3], for monitoring of enzymatic reactions[4] and for process proteomics approaches[5]. In all of these application areas, reliable quantification capability is imperative. With MALDI, reasonable direct quantification can be achieved as long as the cocrystallization process occurs rapidly enough and an internal standard is used[6,7]. Complex sample analysis, however, requires more sophisticated approaches including digestion and labeling of the target proteins[8]. In contrast, the label-free quantification of native proteins is an inherent part of the SELDI process and does not require any additional preparation or labeling (**Fig. 1**).

To demonstrate the practical value of the ProteinChip System, three different types of quantification experiments were conducted using the ProteinChip Reader Series 4000 in the automated mode. Total experiment time was about six hours in each case. Methods and results are briefly summarized here.

Quantification of myoglobin in 1% serum on chromatographic ProteinChip Arrays

A dilution series of myoglobin (16,951.5 Da) in 1% human blood serum was directly applied to chromatographic ProteinChip Arrays (cation exchange, CM10). After a short incubation period, the ProteinChip Arrays were washed twice with buffer and once with distilled water, and then a saturated sinapinic acid solution was applied to the arrays. The subsequent quantification analysis covered more than three orders of magnitude with one single laser protocol. The quantitative response followed a second-order equation, as would be expected with the cocrystallization of increasing amounts of proteins with a constant amount of matrix. Therefore, the slope of the quantification curve was steeper at lower protein concentrations and becomes less steep for higher protein con-

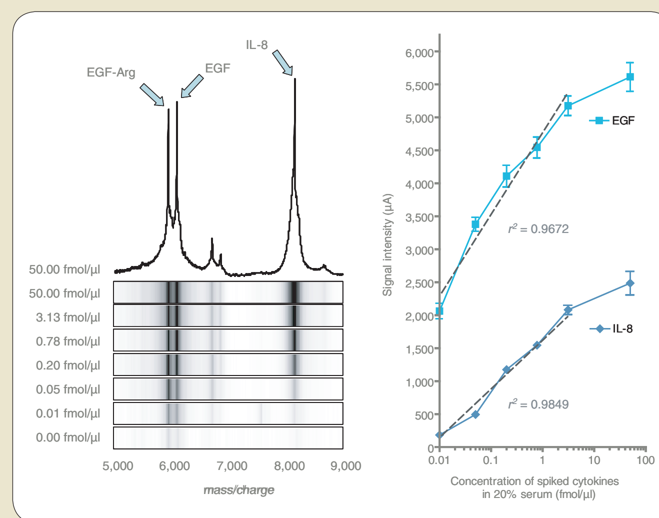

Figure 3 | Simultaneous immunocapture and quantification of EGF and IL-8 from 20% serum. The signal intensities of the EGF peptide and its minus-Arg variant and those of the IL-8 peptide correspond well to the analyte concentrations. For the 50 fmol/µl concentration, the spectrum is given as a line plot as well as a gel plot, an alternative format to visualize the spectra. For the other concentrations, representative spectra are supplied as gel plots only. The graph on the right shows that the dynamic range covers more than three orders of magnitude, with very good linear correlations between signal intensities and logarithmic cytokine concentrations in the range between 0.01 to 3.13 fmol/µl. For EGF, signal intensities of both variants were summed. Data are mean ± s.e.m. from ten dilution series.

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centrations. Nevertheless, at all protein concentrations, a useful quantitative dynamic response is produced. For the ranges 1 to 125 fmol/µl and 125 to 2,000 fmol/µl, a very good linear correlation between signal intensities and protein concentration was achieved (Fig. 2).

Quantification of two cytokines in 20% serum on antibody-coupled ProteinChip Arrays

Two different types of polyclonal antibodies directed against interleukin-8 (IL-8) and epidermal growth factor (EGF) were covalently coupled on preactivated ProteinChip Arrays (RS100). A dilution series in 20% human blood serum of a mixture of recombinant IL-8 (8.4 kDa) and EGF (6.2 kDa) was directly applied to and incubated on the spots. Subsequently, the arrays were washed first with PBS, then with water, and finally 20% CHCA (α-cyano-4-hydroxycinnamic acid) was applied to the arrays.

Both cytokines were specifically captured on the antibody-coupled ProteinChip Arrays (RS100) and analysis revealed very good linear correlation between signal intensities and logarithmic cytokine concentrations in the range between 0.01 and 3.13 fmol/µl. Notably, the EGF-Arg variant (known to appear in serum as a consequence of serum enzyme activities) was likewise captured. This demonstrates the advantage of direct mass determination as part of the analysis process. This type of valuable information cannot be obtained from ELISA-based applications. With SELDI, the user can detect not only the expected specific antigen, but also any covalently modified variants of the antigen, other proteins sharing identical epitopes or any promiscuous ligands (**Fig. 3**).

Monitoring of phosphatase activity on chromatographic ProteinChip Arrays

An alkaline phosphatase solution was added to a peptide solution containing a 1,938.20 Da peptide with one phophorylated serine residue (ISpYGRKKRRQRRRP; kindly provided by Peptide Specialty Laboratories GmbH, Heidelberg, Germany). Aliquots were taken at various time points and analyzed on hydrophilic NP20 ProteinChip Arrays. Spots were washed twice before adding a 20% CHCA

Phosphatase-dependent dephosphorylation of a peptide results in a mass reduction of 80 Da owing to the loss of HPO_3. In order to monitor the dephosphorylation process, we compared the signal intensities of the dephosphorylated (minus 80Da) and the phosphorylated variant over time.Up to 210 min after starting the reaction, the ratio between the signal intensities of the dephosphorylated minus–80 Da variants and the phosphorylated peptides revealed a clear linear increase with time, allowing the rate of phosphatase activity to be determined (**Fig. 4**).

Conclusion

These results demonstrate the capability of the ProteinChip System to address typical experimental needs in biomedical research. A protein from a complex biological solution of 1% blood serum was bound and quantitatively detected on chromatographic arrays, two different cytokines were simultaneously captured and quantified from 20% blood serum by using ProteinChip Arrays precoupled with the corresponding antibodies, and a specific enzyme activity was quantitatively monitored over time.

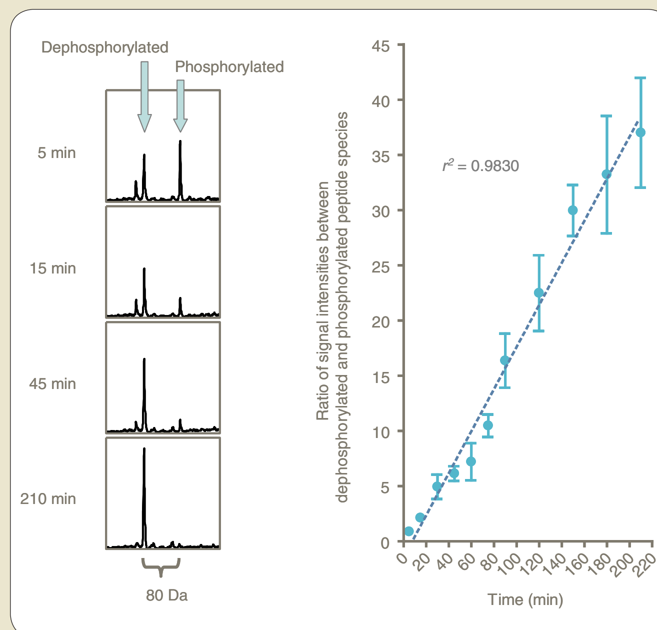

Figure 4 | Quantitative monitoring of phosphatase activity**.** The left panel shows representative spectra for four selected time points (5, 15, 45 and 210 min), with the signals corresponding to the phosphorylated and dephosphorylated peptide variants, respectively. The graph on the right shows the change in the ratio between the signal intensities of both variants over time; that is, the time the added phosphatase could have been active, cleaving the phosphate group from the phosphorylated peptides. The ratios between these two variants show a clear linear dependence on the reaction time between 5 and 210 min after starting the experiment. Data are mean ± s.e.m. from five independent reactions.

By combining selective protein binding with sensitive and quantitative mass detection, SELDI-TOF-MS enables the comparative analysis of virtually any given protein-containing solution in a fast and simple process by using only minute amount of samples.

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