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Use of Lab-on-a-Chip Technology for Protein Sizing and Quantitation

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The performance of the Agilent 2100 bioanalyzer, the first commercial lab-on-a-chip system, and the Protein 200 Plus LabChip® kit is compared with conventional protein analysis techniques such as SDS-PAGE, Lowry, or Bradford. Lab-on-a-chip technology for protein analysis allows for the integration of electrophoretic separation, staining, destaining, and fluorescence detection into a single process, and for it to be combined with data analysis.

The chip-based protein assay allows purity analysis, sizing, and relative quantitation based on internal standards or absolute quantitation based on user-defined standards. The chip-based protein analysis is comparable in sensitivity, sizing accuracy, and reproducibility to SDS-PAGE stained with standard Coomassie. Resolution and linear dynamic range are improved. Absolute quantitation accuracy and reproducibility is improved in comparison to SDS-PAGE and is comparable to batch-based quantitation methods such as Lowry and Bradford. The lab-on-a-chip system has several additional advantages over conventional SDS-PAGE including fast analysis times, reduced manual labor, automated data analysis, and good reproducibility. With such a system, the protein of interest can be tracked during the whole purification procedure, for example, from cell lysates through column fractions to purified proteins.

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Although protein analysis technologies are developing fast, the current standard method for protein sizing is still denaturing sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Many researchers rely on this traditional, labor-intensive, and time-consuming electrophoretic method that has not substantially changed in the past 30 years.[1] With the increasing focus on proteins, there is a strong demand to automate and speed up protein analysis. A significant investment was made to develop more automated methods (e.g., capillary gel electrophoresis), which, however, never replaced SDS-PAGE.

The recent development of microfluidic or lab-on-a-chip analysis systems offers an alternative for protein analysis and has therefore stimulated a lot of academic and industrial research.[2–7] Microfluidic technology allows for the active control of fluids in microfabricated channels that are only a few micrometers in dimension and which have no moving parts. These chips can contain the emulation of pumps, valves, and dispensers for sample handling on the chip, a separation column for electrophoretic separation, and a reaction system. Microfluidic technology aims to integrate several sequential experimental steps into one process to obtain a complete laboratory on a chip. The potential of the methodology has been demonstrated,[2–7] but access to the technology is limited to a few technology centers. Recently, the first commercial lab-on-a-chip analysis system, the Agilent 2100 bioanalyzer (Agilent Technologies Deutschland GmbH), which was developed in collaboration with Caliper Technologies Corp. (Mountain View, CA), was introduced.[8] This system allows for the rapid, automated electrophoretic separation of proteins and the integration of multiple experimental procedures, such

as sample handling, separation, staining, destaining, detection, and analysis into a single process. This paper describes the performance of the chip-based protein analysis in terms of resolution, sensitivity, linear dynamic range, sizing, and quantitation in comparison to conventional SDS-PAGE and protein quantitation methods.

METHODS AND MATERIALS

Material

Human serum albumin (HSA) protein quantitation standards were obtained from Sigma Diagnostics (St. Louis, MO). CE-SDS Protein Size Standard was obtained from BioRad Laboratories (Hercules, CA). All other individual proteins were purchased from Sigma-Aldrich GmbH (Taufkirchen, Germany). Dulbeco's phosphate-buffered saline (PBS) was purchased from Life Technologies GmbH (Karlsruhe, Germany). The Agilent 2100 bioanalyzer and the Protein 200 Plus LabChip kit were obtained from Agilent Technologies (Waldbronn, Germany). All SDS-PAGE reagents and gels were purchased from Invitrogen BV (Groningen, The Netherlands). The digital camera and the imaging software were purchased from Kodak Digital Science (Rochester, NY). Modified Lowry Protein Assay and Coomassie Plus Protein Assay Reagent Kit (Bradford) were obtained from Pierce (Rockford, IL). The Wallac1420 Multilabel Counter used for analysis was from PerkinElmer Wallac GmbH (Freiburg, Germany).

Protein Assay

The chip-based separations were performed on the Agilent 2100 bioanalyzer in combination with the Protein 200 Plus LabChip kit and the dedicated Protein 200 Plus software assay. All chips were prepared according to the protocol provided with the Protein 200 Plus LabChip kit. Each kit includes 25 microfluidic chips and the following reagents: sample buffer, sieving matrix, dye concentrate, and a protein sizing standard. The microfluidic chips consist of two glass layers bond together. One glass layer contains channels etched into it; the other layer contains through wholes that serve as buffer and sample reservoirs and provide access to the channels. The channels of the chip have to be filled with a mixture of a sieving matrix and a fluorescent dye for detection. The channels are filled by pipetting 12 µL of the gel dye mixture into one of the wells and applying pressure with a syringe for 60 seconds. The sieving matrix is a non–cross-linked linear polymer forming dynamic pores to obtain a good resolution. The Protein 200 Plus dye is a red fluorescent dye that interacts with the protein SDS micelles. The gel–dye mixture is also added to the other system wells, where it serves as a buffer reservoir during the separation. The sample preparation is comparable to SDS-PAGE. Sample buffer (2 µL), including lithium dodecylsulfate; a reducing agent (if applicable); and two internal standards, myosin and bradykinin, are added to a 4-µL sample.

The samples are heat denatured at 95–100°C for 3–5 min before loading them onto the chip. The chip is then placed into the bioanalyzer. The bioanalyzer contains sixteen high-voltage power supplies, each of which is connected to a platinum electrode. Once the chip is placed into the instrument, the electrodes touch the liquids in the well, forming an electric circuit. These electric circuits make it possible to move samples from the sample well into the channels and to perform injections into the separation channel. Before each chip run, the optics are focused on the separation channel in the middle of the chip and the chip is equilibrated to 30°C to ensure constant conditions during the separation. Each sample is sequentially separated in the separation channel and detected by laser-induced fluorescence detection (670–700 nm) within 45 sec. The complete analysis of ten protein samples, including sizing and quantitation, takes 25 min (including the start-up phase of the instrument). After completion of the chip run, the data are displayed for each sample as an electropherogram (plot of fluorescence units versus time), as a gel-like image, and in a tabular format.

SDS-PAGE

Gel electrophoresis was performed with 10% and 4–20% pre-cast Tris-Glycine Gels (gel size: 8 cm × 8 cm × 1.5 mm) according to the instructions provided by the manufacturer. An equal volume of Tris-Glycine SDS Sample Buffer (2×) was added to the samples, and they were denatured for 5 min at 95°C before loading onto the gel. The separation was performed for approximately 100 min at constant 125 V. Gels were stained with a noncolloidal Coomassie (R-250) staining kit and destained overnight. A Kodak DC 290 digital camera was used for imaging and analysis was performed with the Kodak 1D Image Analysis Software.

Protein Quantitation

The Lowry and Bradford protein quantitation methods were performed on a microplate according to the

instructions supplied by the manufacturer. The standard curve was generated using six BSA standards with the following protein concentrations: 25, 100, 250, 500, 1000 and 2000 ng/µL. The data points were fitted with a second-order polynomial function. The resulting equation was used to calculate the "unknown" concentration.

RESULTS AND DISCUSSION

The Agilent 2100 bioanalyzer and the Protein 200 Plus LabChip kit were used to analyze different protein samples to verify the performance of the chip-based protein assay with regard to size range, resolution, sensitivity, linear dynamic range, sizing, quantitation accuracy, and reproducibility. Each chip allowed for the sizing and quantitation of ten protein samples ranging in size from 14 to 200 kDa in approximately 45 min, including sample and chip preparation as well as detection and data analysis. The results were displayed by the software in a tabular format, in a gel-like image, and in an electropherogram for each sample. It takes at least 2–3 hr to obtain comparable results with SDS-PAGE. In contrast to SDS-PAGE, no additional manual staining, destaining, imaging, or data analysis steps were required.

The size resolution of the chip-based protein assay was evaluated and compared with the results obtained with conventional SDS-PAGE analysis (Fig. 1) using a protein mixture of eight individual proteins (Table 1) in PBS. The resolution of the chip-based separation was comparable or even better than the resolution achieved using a 4–20% polyacrylamide gel. For example, carbonic anhydrase II (29 kDa) and I (31 kDa), which differ in molecular weight by only 6.5% were almost baseline separated using the chip-based assay. However, they were only partially separated using a 4–20% gradient gel. In addition, three smaller proteins with molecular weights between 17 and 20 kDa are hardly separated with the gradient gel in contrast to the chip-based analysis. Based on additional repeated measurements with a CE-SDS protein sizing standard (data not shown), it was calculated that the chip-based analysis provides a theoretical resolution of at least 10% in molecular weight at half of the peak height within the sizing range of 14–200 kDa. In contrast to 4–20% gradient gels, the resolution of the chip-based separation improves with increasing molecular weight; above 100 kDa a resolution of 5% is achievable. SDS-PAGE, however, allows for optimization of the gel composition to obtain optimal resolution for specific, narrower size ranges. In contrast to SDS-PAGE—which uses a cross-linked gel where the separation depends on the pore sizes—the chip-based analysis system uses a linear polymer gel with dynamic pores, providing an excellent resolution across a large size range. The composition, length, and concentration of the polymer used for the chip-based separation were adjusted during development of the kit to obtain optimal resolution within the 14–200-kDa size range.

The achievable resolution also depends on the characteristics of the proteins within a sample and can be influenced by several factors that affect the migration behavior of proteins. Protein heterogeneity that results in a larger peak or bandwidth can reduce resolution. Proteins that do not migrate according to their theoretical size can also compromise resolution. Both effects are also observed using conventional SDS-PAGE.

To determine the sensitivity of the chip-based protein assay, protein samples containing 100 ng/µL carbonic anhydrase II and 10–3000 ng/µL bovine serum albumin (BSA) in PBS were analyzed (data not shown). The lower detection limit of the chip-based protein assay was determined to be 20 ng/µL BSA in PBS (80 ng in 4 µL of sample) with an approximate signal-to-noise ratio of 3. The sensitivity for standard Coomassie stain (R-250) and colloidal Coomassie stain (G-250) is, as reported by the manufacturers, 50 ng and 10 ng, respectively. The sensitivity of the chip-based protein assay was therefore comparable to that of gels stained with a standard Coomassie stain. However, the sample volume of the chip-based method is limited to 4 µL, whereas SDS-PAGE allows a larger sample to be loaded, thereby increasing the total amount of protein and eliminating the need for preconcentration steps for less concentrated samples.

Due to the electrokinetic injection of the sample into the separation channel on the chip, comparable to capillary electrophoresis, the sensitivity depends on the ionic strength of the sample buffer.[8] If the sample buffer contains a salt concentration lower than that of PBS, a larger amount of protein will be injected into the separation channel, thus enhancing sensitivity. Similarly, increasing the salt concentration in the sample buffer will decrease the sensitivity of the analysis. For example, only 40 ng/µL BSA can be detected in PBS buffer with 0.5 M NaCl with a signal-to-noise ratio of 3. The sensitivity of SDS-PAGE is only slightly affected by the ionic strength of the sample buffer.[8] Furthermore, the sensitivity of both methods, chip-based protein analysis and SDS-PAGE, is affected by the staining efficiency and the peak width of the separated proteins.

As shown in Figure 2, good linearity was achieved for HSA samples ranging in concentration from 150 ng/µL to 2000 ng/µL (correlation coefficient R^2 = 0.9987 and 0.9942). The chip-based protein

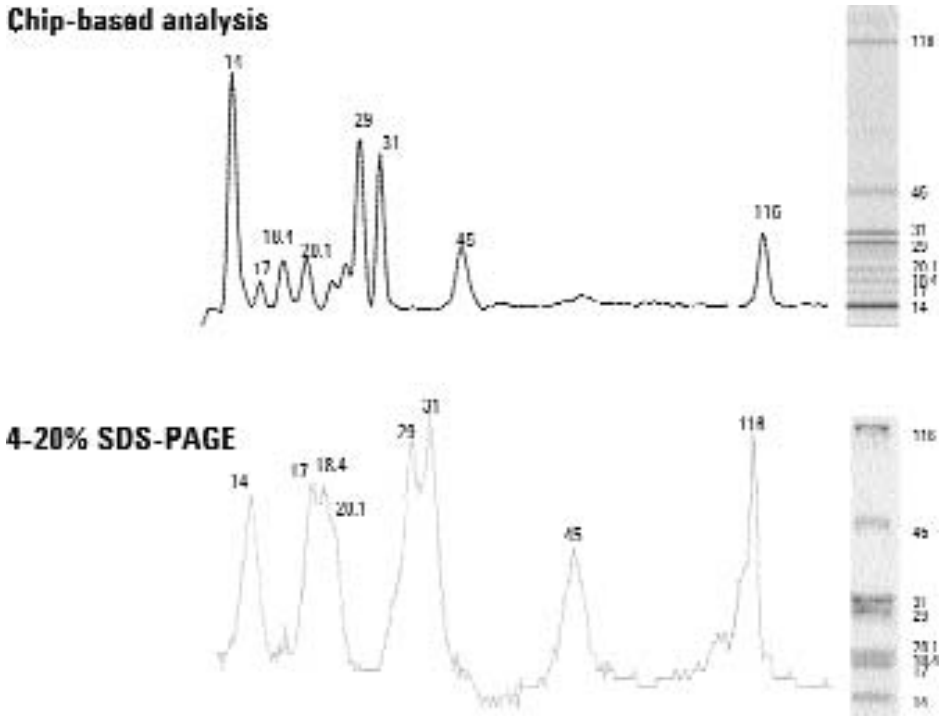

FIGURE 1

Resolution of the chip-based analysis. The size resolution for the separation of a protein mixture of eight different proteins was compared showing the gel-like image and the electropherogram from the chip-based analysis, and the gel image and the gel scan from a 4–20% gradient gel. The molecular weights are shown in kilodaltons.

TABLE 1

Sizing analysis of a protein mixture of eight different proteins in PBS with the chip-based analysis (based on five chips $n = 15$) in comparison to SDS-PAGE (based on 2 gels with $n = 20$)

Protein	Theoretical Size (kDa)	SDS-PAGE (kDa)	SD	Relative SD (%)	Bioanalyzer (kDa)	SD	Relative SD
Lysozyme	14.0	14.0	0.5	3.3	13.8	0.4	2.9
Myoglobin	17.0	17.6	0.5	3.0	16.2	0.6	3.7
β-Lactoglobulin	18.4	18.4	0.6	3.2	18.1	0.5	2.9
Soybean trypsin inhibitor	20.1	19.1	0.6	3.4	20.7	1.0	5.0
Carbonic anhydrase II	29.0	25.3	0.7	2.6	27.5	0.5	1.9
Carbonic anhydrase I	31.0	27.2	0.6	2.1	30.7	0.5	1.5
Ovalbumin	45.0	46.1	1.1	2.3	42.4	0.5	1.2
β-Galactosidase	116.0	116.6	7.8	6.7	118.3	0.6	0.5

assay was determined to be linear in quantitation over two orders of magnitude. This allows, for example, for the detection of a 1% impurity close to a parent peak when checking the purification progress in column fractions. The linear dynamic range that can be achieved with SDS-PAGE is usually smaller.

Protein sizing with the chip-based protein analysis system is performed similarly to SDS-PAGE by running a protein sizing standard on each chip from a designated well. Following the analysis of this sizing standard, the software generates a standard curve of the measured migration time versus the known molecular

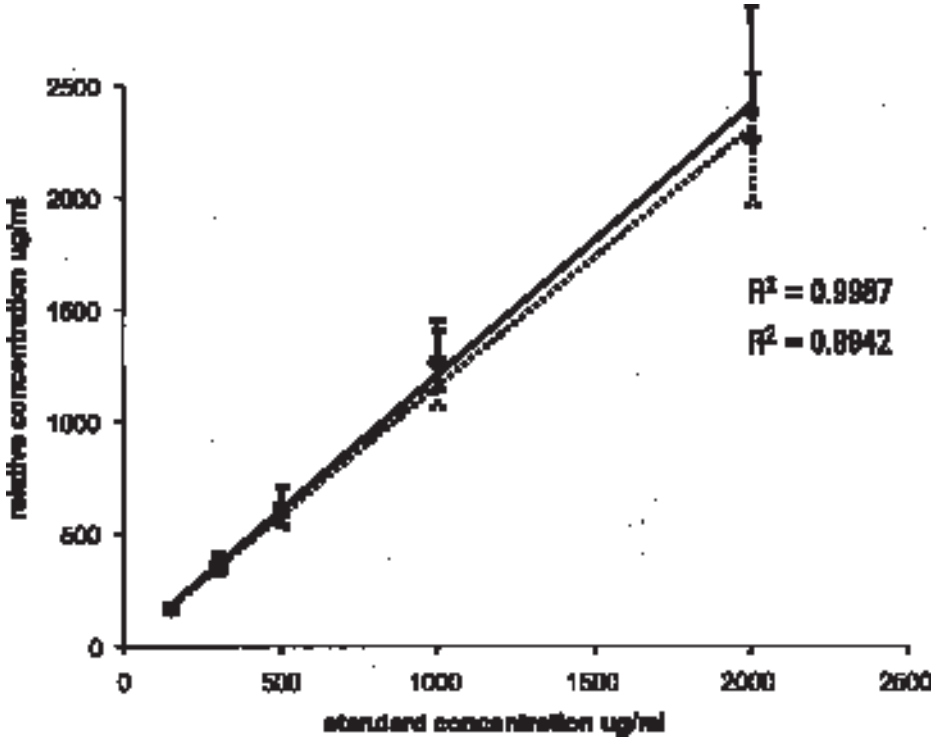

FIGURE 2

Sensitivity and linear dynamic range of the chip-based protein analysis. The measured relative protein concentration of human serum albumin was plotted against the standard concentration as supplied by the manufacturer. A fit with a linear regression was performed and the linear correlation coefficient (R^2) was calculated for two separate measurements with n = 19 each.

weight of each standard protein. This standard curve is used to determine the size of each of the proteins detected within the sample. Internal standards, the lower and upper marker, are included in each sample. They allow for correction of small drifts in migration time and ensure accurate sizing as well as quantitation. The previously used protein mixture (Fig. 1) was used to verify the sizing accuracy and reproducibility of the chip-based protein assay (Table 1). The sizing of the eight different proteins analyzed with the lab-on-a-chip system (5 chips with n = 15) was comparable to both the expected molecular weight and the molecular weight determined using SDS-PAGE (2 gels with n = 20). The sizing accuracy of SDS-PAGE and chip-based analysis depends on the protein characteristics, such as amino acid sequence, isoelectric point, structure, and the presence of certain side chains or prosthetic groups. Sizing accuracy may therefore vary for particular proteins, as some proteins are not migrating according to their molecular weight. The sizing reproducibility (relative standard deviation) of the chip-based protein assay averages around 2.5% compared with 3.3% for SDS-PAGE.

In addition to sizing, the chip-based assay provides a means for relative and absolute protein quantitation. Relative protein concentrations are determined using a one-point calibration, comparing the peak area of the protein of interest with the peak area of the upper marker (myosin) with known protein concentration. The upper marker is used as an internal standard in each sample to correct for differences in injection due to varying salt concentrations and permits determination of the relative concentration independently of the sample matrix. The relative concentration depends on the staining efficiency and can vary from protein to protein. Each of the commonly used total protein quantitation assay methods, such as Lowry, Bradford, or BCA assays, exhibits some degree of variation in staining efficiency when assaying different proteins. Protein stains used for SDS-PAGE show this effect as well. The staining efficiency depends upon the characteristics of the spe-

TABLE 2

The Quantitation Accuracy and Reproducibility for the Different Quantitation Methods*

Method	Rel. conc. (µg/ml)	Rel. SD (%)	Error (%)	Absolute conc. (µg/ml)	Rel. SD (%)	Error (%)
Chip-based protein analysis	912	9	22	856	7	14
Lowry				749	4	1
Bradford				895	6	19
SDS-PAGE				995	13	25

*Chip-based protein analysis, Bradford and Lowry assays, and SDS-PAGE: accuracy and reproducibility were determined using BSA at a target concentration of 750 µg/mL as sample and a calibration curve with six different BSA concentrations (n = 5 for all methods).

cific protein—such as amino acid sequence, isoelectric point, hydrophobicity, and other physicochemical properties—and upon its interaction with the used dye. The detection of the lab-on-a-chip system is based on laser-induced fluorescence of an intercalating dye, which interacts with the protein/SDS complexes. Similar to other protein staining methods, it is also affected by staining efficiency.

As demonstrated in Table 2 with the analysis of a BSA sample, more accurate, absolute quantitation can be obtained by using a calibration curve generated with the same protein. A protein calibration feature in the software of the chip-based analysis system allows for the performance of absolute protein quantitation based on user-defined standards with known protein concentration. The software automatically generates a protein calibration curve to determine the absolute concentration of actual samples within the same chip. Absolute protein concentrations as well as protein purity and size are determined in a single experiment. The quantitation accuracy and reproducibility of the chip-based protein analysis are comparable to that achieved with the batch-based Lowry and Bradford assays (Table 2). However, the quantitation accuracy and reproducibility achieved by using SDS-PAGE is worse. Both chip-based protein analysis and SDS-PAGE allow for the quantitation of individual proteins within a sample; the batch-based assays, such as Lowry and Bradford, only allow for the determination of the total protein concentration. Furthermore, the chip-based protein assay is compatible with most of the commonly used protein sample buffers, including a wide variety of reagents such as 1% SDS, 100 mM dithiothreitol, or 500 mM imidazole, whereas the batch-based assays are more susceptible to those additives.

CONCLUSION

The lab-on-a-chip system is an ideal tool for the analysis of proteins, as it provides information on size, concentration, and purity in a single assay. The performance of this system in terms of sizing and sensitivity is comparable to that of conventional SDS-PAGE in that linear dynamic range and resolution are superior, but analysis times are greatly reduced. Additional advantages are ease of use, automated separation, detection and data analysis, good reproducibility, digital data, and significant reduction of hazardous waste. Furthermore, the hardware and software of this system can be used with a variety of other reagents and chips for the analysis of DNA, and RNA. In addition, the lab-on-a-chip system allows for simple flow cytometric analysis of protein expression in eukaryotic cell populations (e.g., monitoring transfection efficiency by green fluorescent protein detection[9] or antibody staining of intracellular or cell surface target proteins.[10])

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