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TEMPERATURE AND DENATURING SUBSTANCES INFLUENCE ON LAB-ON-A-CHIP ELECTROPHORESIS

UTICAJ TEMPERATURE I SREDSTAVA ZA DENATURISANJE NA ELEKTROFOREZU VRSTE »LABORATORIJA NA ČIPU«

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Summary: Qualitative and quantitative determination of proteins in different biological fluids is of great significance in medicine, due to their importance in diagnosis and treatment of some diseases. Nowadays, different methods for protein analysis are available. Lab-on-a-chip electrophoresis is a relatively new technique, based on microfluidics, which allows samples of biological fluids to be analyzed within a microchip. This paper describes the optimization of performance of the chip-based protein analyses in serum samples from patients with different neurological disorders. Using microchip technology, serum proteins with the molecular mass from 4.5 to 240 Kb were separated and sized. The fluorescence detection method in the analysis was used to follow the influence of the temperature and the type and concentration of denaturing substances on the electrophoresis protein profiles. It was noted that, depending on incubation temperature and denaturing substances, different electrophoresis patterns can be obtained from the proteins of one specimen. Significant change of the fluorescence intensity was observed when different incubation temperatures were used, probably due to fluorescence quenching. In some cases, the band intensity was changed several times. Lab-on-a-chip electrophoresis is a very efficient method for the separation and determination of different serum proteins in a very short time. However, to obtain comparable results for the analysis, the denaturing agent concentration and temperature must be observed and maintained carefully.

Keywords: electrophoresis, fluorescence quenching, lab-on-a-chip, neurological diseases, protein analysis, serum samples

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Kratak sadržaj: Zbog važnosti proteina u dijagnostici i lečenju nekih bolesti, njihovo kvalitativno i kvantitativno određivanje u biološkim tečnostima od velikog je značaja u medicini. Postoje različite metode za analizu proteina. Elektroforeza vrste »laboratorija na čipu« relativno je nova tehnika, zasnovana na mikrofluidici, što omogućava analiziranje uzoraka bioloških tečnosti u okviru mikročipa. U radu je opisana optimizacija postupka analize proteina na bazi čipa u uzorcima seruma pacijenata sa različitim neurološkim poremećajima. Pomoću tehnologije mikročipova, razdvojeni su i izmereni proteini iz seruma sa molekulskom masom 4,5–240 Kb. Korišćenjem pri analizi metode fluorescentne detekcije praćen je uticaj temperature i tipa i koncentracije sredstava za denaturisanje na elektroforetske profile proteina. Uočeno je da se, u zavisnosti od temperature inkubacije i sredstava za denaturisanje, od proteina istog primerka mogu dobiti različiti elektroforetski obrasci. Primećena je i značajna promena intenziteta fluorescencije pri različitim temperaturama inkubacije, verovatno usled gašenja fluorescencije. U nekim slučajevima je po nekoliko puta menjan intenzitet trake. Elektroforeza vrste »laboratorija na čipu« je vrlo efikasan metod za separaciju i određivanje različitih proteina u serumu u veoma kratkom vremenskom roku. Međutim, moraju se pažljivo posmatrati i održavati koncentracija i temperatura sredstva za denaturisanje, da bi se dobili uporedivi rezultati za analizu.

Ključne reči: elektroforeza, gašenje fluorescencije, »laboratorija na čipu«, neurološka oboljenja, analiza proteina, uzorci seruma

Introduction

Protein profile in patients with neurological diseases is one of the crucial diagnostic criteria, as well as a tool in the prognosis and treatment of the disease. Therefore, it is of great importance to analyze the protein content and characteristic profile in the patients suffering from conditions such as multiple sclerosis, epilepsy, pareses, dementias, brain strokes

and disorders in the function of the blood-brain barrier. Conventional electrophoresis methods, mainly sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and disc-PAGE are widely used in performing the protein profiling (1). However, since a rapid and precise method is needed, improvements of the techniques are daily investigated and included in the analysis process.

Lab-on-a-chip electrophoresis is a relatively new improved technique that can perform protein profiling within the confines of a microchip. This technique integrates several procedures, such as sample handling, separation, staining, destaining, detection and analysis, all in a single process (2–4). Miniaturizing the laboratory onto a chip is considered to be a step forward, due to the number of advantages, mostly in the time and labor frame of work. It requires smaller amounts of material, therefore conserving both sample and reagents, which is important in clinical analyses where sample quantities are often limited. The small scale of the chip increases the speed with which samples are processed and also, since the manual work is limited in comparison to conventional electrophoresis, results are more accurate and reproducible.

Optimization of the conditions for handling samples is of great importance when clinical application of the technique is required. One of the basic parameters that should be optimized, especially when proteins are considered, is the incubation temperature and denaturing substances. Proteins show different bonding affinity to the denaturing agents when incubated at different temperatures. Despite of the most abandoned denaturing reagent used, sodium dodecylsulphate (SDS), additional agents can be used, such as β-mercaptoethanol (BME) and urea. Their concentration differs in relation to the sample protein concentration and the temperature of incubation. These denaturing agents, as well as temperature increases lead to changes in the fluorescence detection signal (5).

Due to the mentioned requirements, and in the process of achieving better and faster protein profiling, we have optimized and studied these parameters and their influence on the patients' serum protein profiles using lab-on-a-chip electrophoresis.

Materials and Methods

Materials

Serum samples were obtained from the biochemical laboratory in the Clinic of Neurology (Faculty of Medicine), and were labeled according to the Declaration of Helsinki ethical committee. High molecular mass (HMW) and low molecular mass (LMW) protein standards were obtained from BioRad Laboratories. Denaturing agents, SDS, β-mercaptoethanol (BME) and urea were obtained from Sigma-Aldrich. Lab on-a-chip electrophoresis was carried out on the Agilent 2100 Bioanalyzer from Agilent Technologies, Santa Clara, USA. Protein 200 Plus LabChip kit was used in the analysis.

Methods

Serum samples were collected from whole blood by vein punction, and stored at –80 °C until analyzed. Before handling the serum samples, total protein, albumin and immunoglobulin G (IgG) concentrations need to be measured. The total protein concentration in the serum was determined by a spectrophotometric method, using the Alcyon 2100 Bioanalyzer. Turbidimetry was used to analyze albumin and immunoglobulin according to the prescript procedure. All chips were prepared according to the Agilent protocol provided. The samples were set using the proposed procedure, as well as the optimized procedure. The gel and the gel dye mix were prepared as proscribed in the Agilent manual (5, 6).

When optimization of the denaturing agent was performed, samples were prepared using 79 μL deionized water, 4 μL sample and 7 μL of BME. Protein standard was included in every run, therefore providing comparison between different runs. The influence of the additional denaturing agents (SDS and urea) was determined using the same procedure of sample preparation, and the signals of the proteins were observed. Afterwards, different concentration of the denaturing agent was used in order to obtain the optimal sample: denaturing agent ratio. When the temperature influence was studied, sample incubation was done at five different temperatures: 5 °C, 22 °C (room temperature), 37 °C, 60 °C and 90 °C. After sample preparation, samples were loaded onto a chip. The chip was placed in the bioanalyzer and run. In the process of electrophoretic separation in the bioanalyzer, the electric current provides ideal conditions for staining and destaining within 45 s per sample. The analysis chamber itself is heated to 30 °C to ensure the constant conditions. After the separation, the signals are detected by laser-induced fluorescence detection (670–700 nm) (6–8). The Protein 200 Plus software assay was used to obtain the complete numerical analysis. The reproducibility and statistical analysis were performed using Statsoft Statistica v7.0.61.0.

Results and Discussion

Lab-on-a-chip analyses have proven to be more precise and less troubleshooting when analyzing complicated protein mixtures, in comparison to conventional electrophoresis. The analyses of serum samples from the patients were performed in order to determine their protein profile. Both control group samples

and patient samples were analyzed and compared to the protein standard *(Figure 1)*.

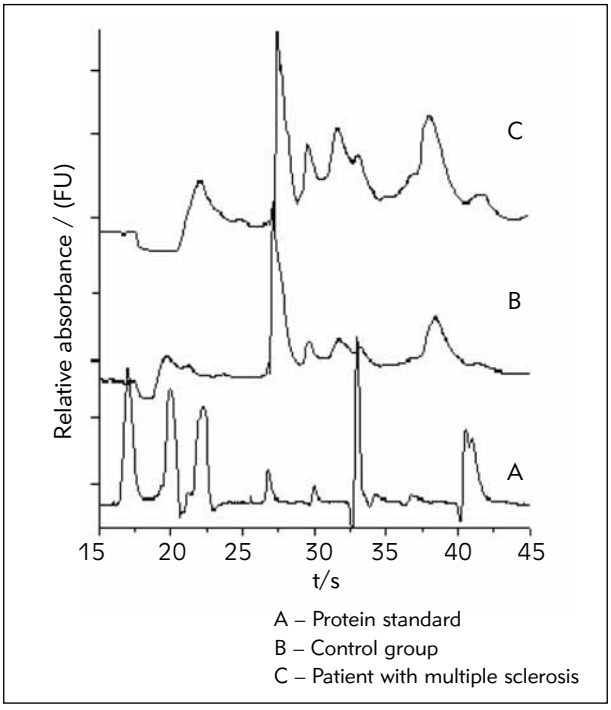

Figure 1 An example electropherogram of serum protein profiles.

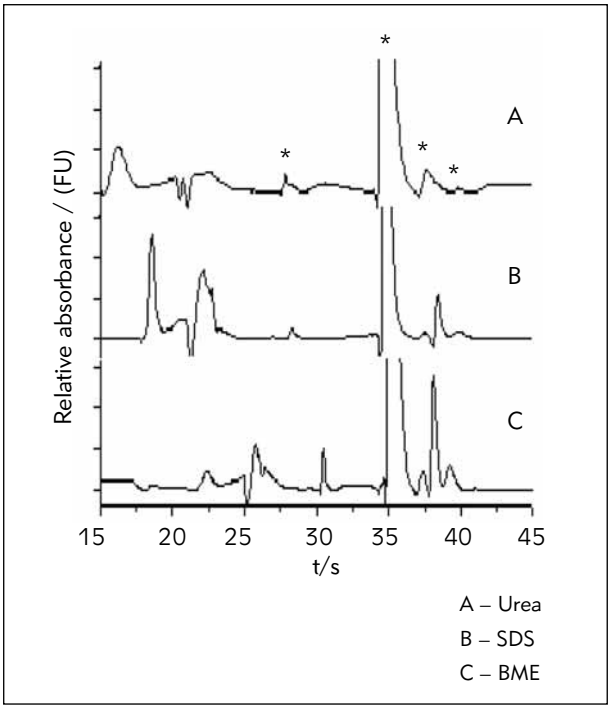

Figure 2 The influence of the nature of denaturing agent on the electrophoresis (the peaks were analyzed, and the most prominent changes in the influence noticed, are assigned with *):

Determination of the protein molecular mass was done, as well as band-shape and peak analysis. Protein sizing with the chip-based analysis was performed by running a protein standard consisting of 10 proteins (HMW plus LMW standard in optimal ratio) and was analyzed in the terms of examining reproducibility (data not presented). Results have shown that the protein profile differs in samples with neurological diseases in the range of appearance of different additional protein fractions. Concentration changes have also been noted for the immunoglobulin fractions in the patients suffering from system disorders, and for the alpha-globulin fraction in patients with inflammation diseases.

We used this method to analyze the influence of several denaturing agents and their concentration on the sample preparation. The effects on the electrophoretic protein profile were observed and explained using the method of laser-fluorescence quenching. The samples were prepared under reducing conditions, using SDS, urea and BME as reducing agents. It was noted that incubation with BME gives best resolution (*Figure 2*).

Further, BME concentration in the sample (total protein: denaturing agent) was optimized. We have used different concentrations varying in ratios TP: BME (1:1; 1:1.5; 1:2; 1:2.5: 1:3; 1:3.5 and 1:5). The results have shown that the optimal ratio for serum sample analysis is 1:3.5 (*Figure 3*). Additional increase in the BME concentration does not influence the resolution and signal of protein peaks.

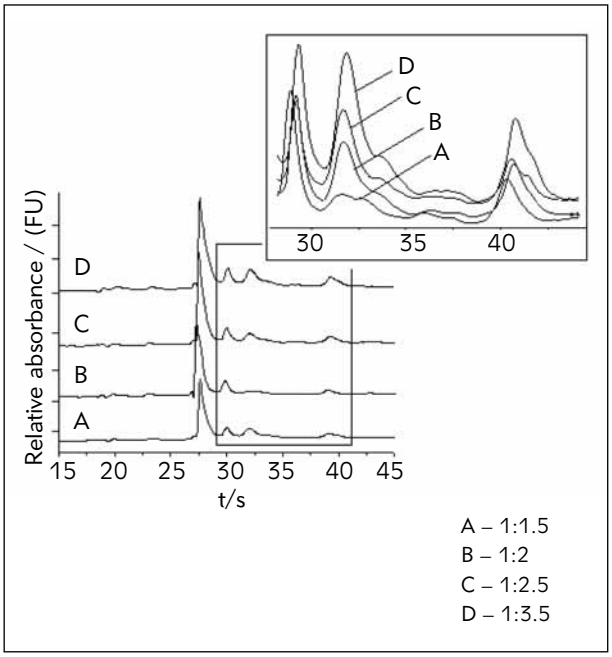

Figure 3 Determination of the optimal concentration serum: BME ratio in protein detection. (Some characteristic samples are presented).

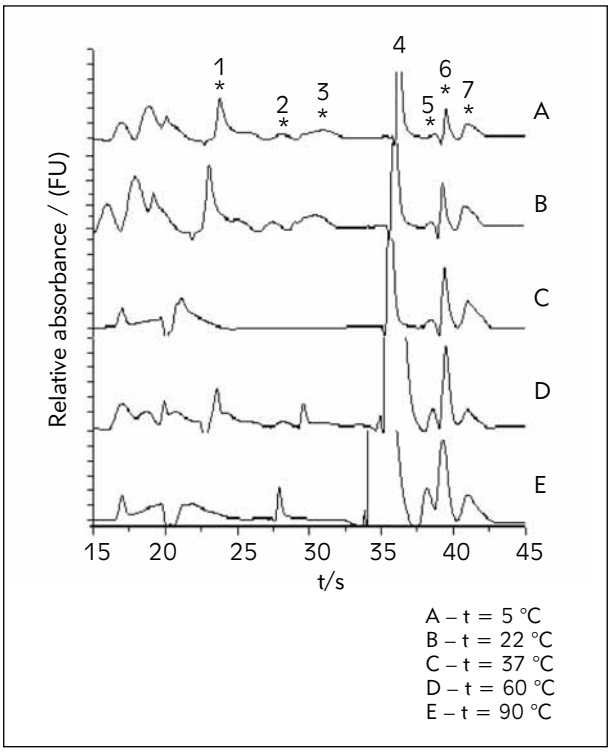

Figure 4 Influence of the temperature of incubation on the lab-on-a-chip electrophoresis serum protein analysis (the peaks analyzed, the most prominent changes in the influence noticed, are assigned with * and are numbered).

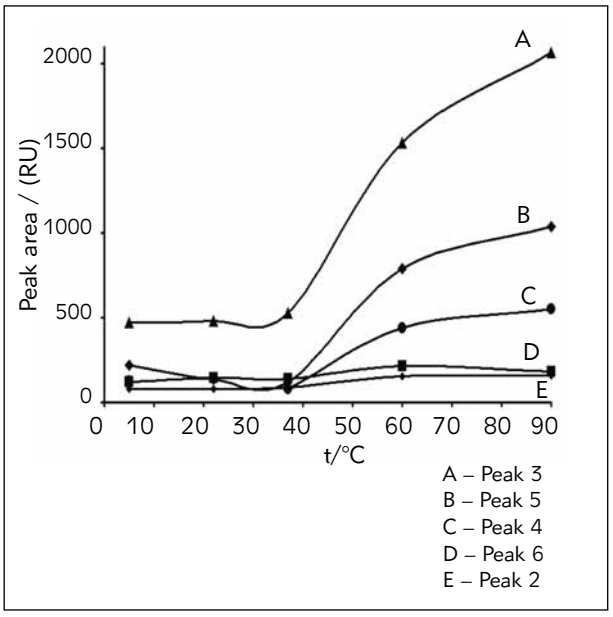

Figure 5 Temperature dependence of the peak band area. Five most intensive protein peaks were included in the analysis (noted on *Figure 4*).

It is noted that the fluorescence signal increases when the denaturing agent concentration increases to a certain level. This effect can be explained when the fluorescence mechanism is considered. In the native state, the fluorescence from the dye molecules is severely quenched. When proteins are denatured they unfold, and the fluorescence increases. Such a dramatic change is measurable even at the single molecule level, permitting the study of folding dynamics of protein molecules in real time (8, 9).

When analyzing protein mixtures, temperature is a very important parameter due to its effects onto the protein conformation and affinity to bind with the dye, and therefore to increase the signal of detection. For providing denaturing conditions, sample preparation should be done by boiling with the denaturing agent. In Disc-PAGE, however, it is noted that lower temperatures provide better results. We have analyzed this effect at 5 temperatures of incubation using the optimized denaturing agent, BME, in the optimal concentration within sample preparation. It was noted that the optimal temperature for generating denaturing conditions is 90 °C (*Figure 4*).

Five peaks with notable changes in intensity (peak 2, 3, 4, 5 and 6) were observed, and the fluorescence quenching effect was noticed (*Figure* 5).

Significant changes in the peak intensity were observed. The effect is similar to when denaturing agents are considered. Because of the samples' complicated structure and the three-dimensional structure of the protein constituents, the dye molecules are in close proximity with each other and their fluorescence is severely quenched. When heated and denatured, proteins unfold, swell and reduce the proximity among dye molecules, thus leading to an increase of the fluorescence.

Lab-on-a-chip is more efficient technique when analyzing complicated protein mixtures in comparison to conventional electrophoresis. However, its usage in routine analyses is still limited, due to the need of an optimized method which is easy to handle, cheap and follows certain procedure. The serum proteins are a complicated mixture and are very sensitive to changes in sample temperature and denaturing agents. Significant change of the fluorescence intensity is observed probably due to fluorescence quenching. In some cases, the band intensity was changed several times. Therefore, further analyses are necessary in order to ensure implementation of the technique in routine laboratory practice.

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References

1. Mitrevski A. Prilog kon ispiyuvanjeto na proteini vo likvor so elektroforeza, Doktorska disertacija, Skopje 2000.
2. Kuschel M, Neumann T, Barthmaier P, Kratzmeier M. Use of lab-on-a-chip technology for protein sizing and quantification. J Biomol Tech 2002; 13: 172–8.
3. Effenhauser CS, Paulin A, Manz A, Widmer M. High--speed separation of antisense oligonucleotides on a micromachined capillary electrophoresis device. Anal Chem 1994; 66: 2949–53.
4. Woolley AT, Mathies RA. Ultra high-speed DNA fragment separation using microfabricated capillary array electrophoresis chips. Proc Natl Acad Sci USA. 1994; 91: 11348–52.
5. Desai S, Barthmaier P. Evaluation of albumin removal using the Agilent 2100 bioanalyzer. Agilent tech. 5988–9911EN, 2003.
6. Bousse L. Protein sizing on a microchip. Anal Chem 2001; 73: 1207–12.
7. Schmut O, Horwath-Winter J, Zenker A, Trummer G. The effect of sample treatment on separation profiles of tear fluid proteins: Qualitative and semi-quantitative protein determination by an automated analysis system. Graefe's Archive for Clinical and Experimental Ophthalmology 2002; 240: 900–5.
8. Fluorescence spectroscopy: A tool for Protein folding/ unfolding study PC 3267. Jan 2007.
9. Zhuang X, Ha T, Kim HD, Centner T, Labeit S, Chu S. Fluorescence quenching: A tool for single-molecule protein folding study. PNAS 2000; 97, 26: 14241–4.

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