SENSITIVE METHOD OF CASPASE-3 DETECTION IN SINGLE STEM CELL

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Summary

Caspases are involved in physiological process (e.g. cellular differentiation) but also can cause serious disorders. It is getting necessary to develop sensitive, miniaturized and fast methods amenable to analyze small amounts of samples. Luciferin/luciferase chemi-luminescence reaction is one of the methods of caspase-3 detection. We have developed a miniaturized device enabling detection of caspase-3 and quantitation just in femtogram level (10^{-15} g) in single apoptotic human stem cells (neural crest derived). The technology is based on the specific cleavage of modified luciferin by caspase-3, emissions of photons and their detection by photomultiplier tube working in the photon counting regime.

1. Introduction

Inhibition of apoptosis leads to cancer or autoimmune diseases, induction of apoptosis is responsible for neurodegenerative disorders such as Parkinson's disease Huntington's disease, multiple sclerosis or Alzheimer's disease. Considering this facts make analysis of caspase-3 in single cells essential for further research, clinical practice and also for discovery of new potential drugs. There are various techniques for caspase-3 detection: bioluminescence, fluorimetry (including fluorescence resonance energy transfers or quantum dots), colorimetry, atomic force microscopy, electrochemistry, magnetic resonance imaging and nuclear imaging[1]. Bioluminescence is mostly based on luciferin/luciferase system and detection of emmited photons by a photomultiplier tube. The aim of our study is to improve sensitivy of caspase-3 enabling its detection in individual stem cell.

2. Experimental

Cells (neural crest derived) were treated by camptothecin, a cytostatic alkaloid, for a period of ten hours to induce apoptosis[2]. The portion of 1–5 stem cell was captured and transferred by a micromanipulation system into a detection capillary filled with 2 µl of the reagent (Caspase-Glo™ 3/7 (Promega)) (Fig. 1). The amount of caspase-3 was detected by photomultiplier tube (PMT) working in the photon counting detection regime (Fig. 2).

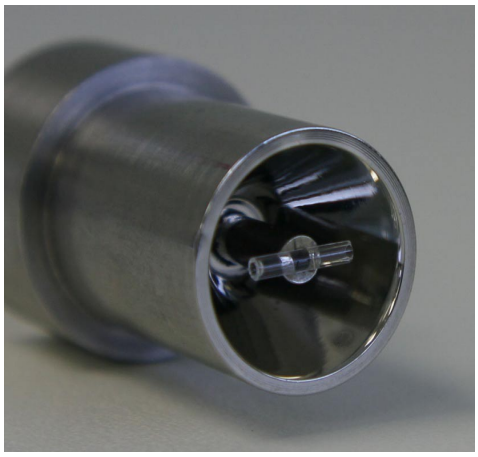

Fig. 1. **Detection capillary filled with 2 µl Caspase-Glo™ 3/7 reagent placed inside reflective chamber**

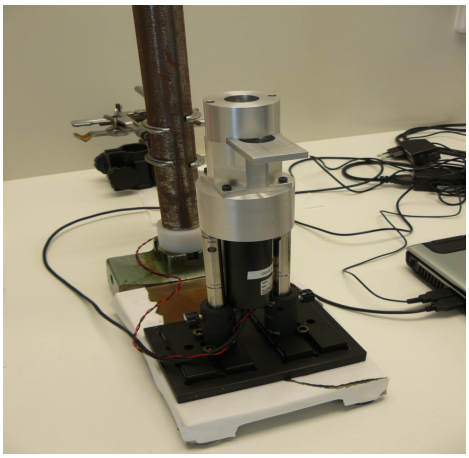

Fig. 2. **Detection chamber above PMT window**

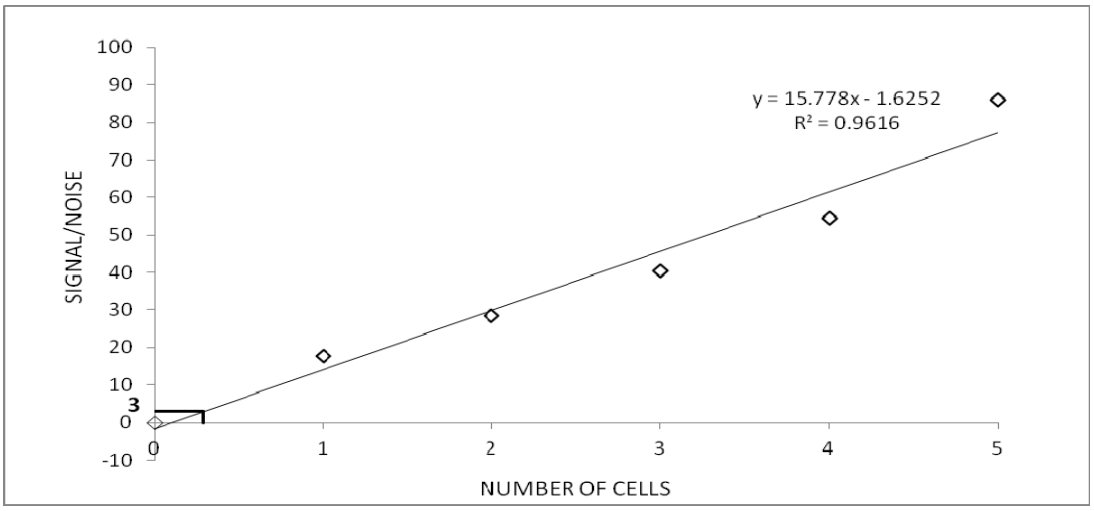

Fig. 3. **Dependence of signal to noise on number of stem cells.** Limit of detection is approximately half of the amount of caspase-3 in a single apoptotic stem cell

3. Results and discussion

The steady signal reached after 50 minutes of cell lysis is proportional to the amount of caspase-3. A decrease of the reagent volume due to evaporation is negligible after one hour of measurement. Considering previous bioluminescence methods there was high signal of background. The prerequisite of a higher sensitivity of our system, when compared with commercial devices, is a low background signal due to the small amount of Caspase -Glo™ 3/7 reagent and implementation of photon counting detection method.

4. Conclusions

The detection limit achieved in the miniaturized device is much lower than the amount of caspase-3 in single apoptotic stem cells. We proved high sensitivity of caspase-3 detection at the level of femtograms.

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INTEGRATED MICROFLUIDIC DEVICE FOR DROPLET MANIPULATION

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Summary

Droplets based microfluidic systems have a big potential for the miniaturization of processes for bioanalysis. In the form of droplets, reagents are used in discrete volume, enabling high-throughput chemical reactions as well as single-cell encapsulation. Microreactors of this type can be manipulated and applied in bio-testing. In this work we present a platform for droplet generation and manipulation by using dielectrophoresis force. This platform is an integrated microfluidic device with a dielectrophoresis (DEP) chip. The microfluidic device generates microdroplets such as water in oil emulsion.

1. Introduction

Droplets in miniaturized microfluidic systems such as water in oil emulsion are promising for use as well-defined and confined microreactors[1]. The benefits of this system are a large reduction in the volume of reagent in each droplet, the small amount of samples required, and the miniaturization of the equipment itself, reduced cost and reaction time[2]. By reducing the volumes it is possible to enhance the speed of assay. Mixing of reagents in droplet has been proved to be achieved within few minutes and it is much easier in droplets than in continuous microflows. Multiple emulsions or structured drops can offer even more functionalities, such as cell encapsulation for targeted delivery or effective high-throughput screening, which requires a much higher degree of control, with access to individual droplets. Such control can be achieved using microfluidic technology[1], which enables the formation of uniform drops, the drop manipulation[3] and the mixing of small volumes. However, manipulation of drops in the microfluidic device is essential. This work aims at examining the possibility to manipulate the microdroplets by using dielectrophoresis force. Integrated microfluidic device with a DEP chip generates surfactant stabilized droplets using an inert oil as the continuous phase. The control circuit enables us to monitor *in situ* the change of motion direction of a droplet.

2. Experimental

Water in oil emulsion was generated using a T-junction droplet generator chip fabricated as described in the literature. The chip was inserted into the holder and connected to two syringe pumps using bare-fused silica capillaries. Two 250 μL glass syringes were filled with aqueous and continuous phases.

3. Results and discussion

Emulsion formation. We have tested different oil phases such as mineral and silicone oils; decane, dodecane and decalin. In order to stabilize an emulsion, we used a combination of surfactants selected on the basis of hydrophilic lipophilic balance (HLB) values. Hence we have chosen commercially available surfactants Span 80 and Triton X 100. According to the results of the previous studies[5], we investigated three different surfactant mixtures of Span 80 and Triton X 100 in decane. It was demonstrated that the ratio of Span 80 and Triton X 100 98:2 is optimal under the given conditions and these components were added into the emulsion. The studies permitted to determine conditions of emulsion formation. The samples of oil phase such as decane, dodecane, decalin, mineral oil and silicon oil were tested with a more suitable ratio Span 80-Triron X 100 found as mentioned above. The results are presented in the Fig. 1, from which we can derive that the system decalin/Span 80-Tritox X100 has no coalescence. This system is characterized by a stable emulsion for one month. It was chosen for future experiments. The size of water drops was controlled by adjusting flow rates of oil and water with syringe pumps. We have measured the droplet size using an optical microscope. The size of the generated droplets was in the range of 10–50 μm.

Droplet generation. In order to generate emulsion (w/o) we fabricated a microfluidic chip which was connected to microfluidic droplet generation system. Two syringe pumps were filled with continuous and aqueous phases. We conducted the emulsification using a T-junction geometry microfluidic device. The width of the nozzle in the device was 75±1 μm. All dimensions of the microchannels were maintained identical. In the emulsification process, we used the optimized values and ratios of the continuous and aqueous phases. Emulsion was collected by passing the outlet flow from the chipset to the array.

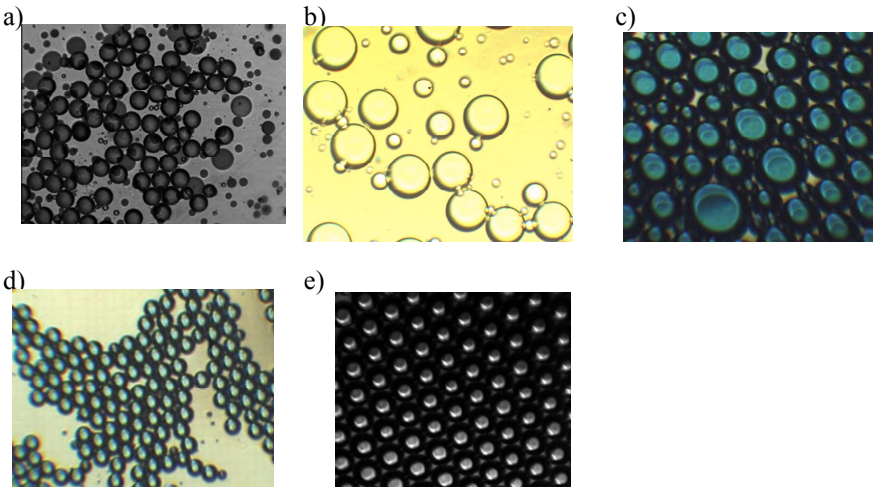

Fig. 1. **Optical micrographs of (a) mineral oil; (b) silicon oil; (c) decan; (d) dodecan; (e) decalin water in oil emulsion in the aqueous phase**

Droplet manipulation. The emulsion was collected onto the array (Fig. 2) and covered with an ITO-glass using parafilm "M" as a spacer. We applied AC voltage 25V at frequency ranging from 50 Hz to 1 MHz across the electrodes. Under these conditions we observed positive dielectrophoresis of water droplets. Droplets were attracted to the edges of electrodes, where the gradient of the electric field is the highest. We have also observed coalescence of droplets induced by AC current. This undesirable phenomenon needs to be avoided. One of possible ways to prevent it is to have sufficient distances between droplets. This could be achieved by using inlet capillary for the emulsion and pulling droplets one by one directly from the capillary. Then the droplets will be manipulated separately.

4. Conclusions

The described platform – a microfluidic device with an integrated dielectrophoresis chip for droplet manipulation – is an essential component for high-throughput bioassay. The generated droplets were placed onto the electrode array where directions of their motion could be influenced through the DEP force. The manipulation flexibility can be further increased by using higher fields, thinner electrodes or different layout of the electrode array. Applying this system for the future work involves single cell encapsulation and detection by optical and mass spectrometric means[4].

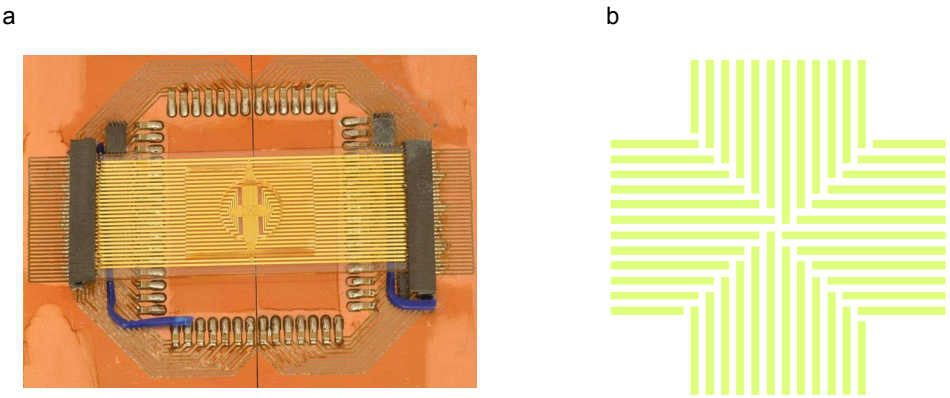

Fig. 2. **a) Schematic top view of the microelectrode array connected to the generator; b) layout of the four-segment microelectrode array which enables inducing motion perpendicular to the electrodes**

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HIGH-THROUGHPUT MALDI TOF MASS SPECTROMETRY IMAGING

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Summary

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) imaging of biological tissues took an influential place among imaging techniques during the past decade[1–3]. It has been successfully exploited in the field of biomarker discovery, drug and metabolites analysis, brain tissue studies, artwork inspection, and other analytical fields. One of the drawbacks of the technique preventing its spreading to the clinical practice is a difficult imaging automation and a low speed of the MS image acquisition. A novel high-speed MS imaging sampling technique employing scanning of the desorption laser beam introduced earlier[4] is investigated.

1. Introduction

Typical MALDI MS imaging experiment comprises a series of MS signal acquisitions from a surface of matrix-covered tissue, data reading, processing and storage and finally translations between measured points (pixels). Aside from the sample preparation; cryosectioning, washing, drying and matrix application – which can be done parallelly to the image acquisition; the MS imaging time in modern high-repetition laser instruments is limited by the data processing and the target translation between the pixels executed by linear motorized stages. To reduce this bottleneck, we developed a sampling approach utilizing parallel data processing and fast laser beam scanning partially replacing the stages translation.

2. Experimental

2.1. Laboratory-built mass spectrometer

Laboratory-built axial mass spectrometer is equipped with a 355 nm diode-pumped frequency-tripled Nd:YAG laser (DTL-374QT Laser system; Lasers Innovations) efficiently working at frequencies up to 4 kHz. The key instrument feature for the high-throughput MS imaging is a fast precision scanning mirror (6810P; Cambridge Technology) capable of redirecting desorption laser beam on a sub-millisecond time scale. Scanning mirror can move the laser beam few millimeters across the sample, efficiently replacing the translation stage movements.

2.2. Model sample preparation

Model sample for evaluating the performance of laboratory-built instrument; 200 μL of matrix-peptide mixture (20 mg/mL DHB, 10 μM ACTH, 40/60 v/v MeOH/H_2O) was sprayed on the MALDI plate from the distance of 8 cm through a 7-mm circular mask using an airbrush GRAFO T1 (Harder & Steenbeck Airbrush, Norderstedt, Germany).

3. Results and discussion

The MS imaging performance of the laboratory-built instrument with Nd:YAG laser (1–4 kHz) and fast scanning mirror was compared to the performance of the

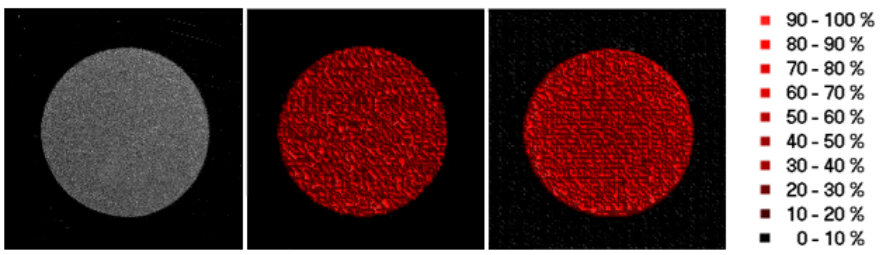

Fig. 1. **(A) Model sample photograph. MS images of the model samples at *m*/*z* = 2466.2±1 recorded in the reflector mode using (B) AutoFlex™ and (C) 4-kHz laboratory-built instrument with 1-mm laser beam scanning**

Table I
Comparison of MS imaging time of 1-cm^2 area (100 μm resolution, 100,000 pixels, 100 laser shots per pixel) with AutoFlex™ Speed and the laboratory-built MS

Device	Sampling mode	f [kHz]	t_{trans} [ms]/ pixel	t_{acq} [ms]	t_{proc} [ms]	t_{cycle} [ms]	t_{total} [min]
AutoFlex™	Stage translation	1	–	–	–	465	77.50
Lab-built MS	Stage translation	1	117	100	17.0	240	39.95
Lab-built MS	1-mm laser scanning	1	22	100	17.0	139	23.16
Lab-built MS	Stage translation	4	117	26	17.0	160	26.70
Lab-built MS	1-mm laser scanning	4	22	26	17.5	66.5	11.08

commercial TOF mass spectrometer (AutoFlex™ Speed) on model sample described above. The 1-cm^2 imaged area consisting of 10,000 measurement points with the spacing 100 μm was recorded with both instruments in traditional rastering mode and in laser beam scanning mode with laboratory-built instrument. The resulting images are showed in Fig. 1. Recorded MS imaging times are summarized in Table I.

4. Conclusions

A new sampling approach, laser beam scanning sampling, was introduced to MALDI MS imaging. In this mode, MS image is acquired with combined laser beam scanning and stage translation reducing the sample translation bottleneck of the MS imaging. The sampling mode utilizing the high-speed motion of the laser beam was shown to significantly outperform translation (rastering) mode, thus decreasing the imaging time. Equipped with the scanning mirror, the laboratory-built axial MALDI TOF MS instrument utilizing 4-kHz UV laser recorded a 100 × 100 pixel MS image in ~11 minutes using 100 laser shots per pixel, improving the speed of the analysis by a factor of ~7 compared to the commercial instrument.

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OPTIMIZATION OF *N*-GLYCOPEPTIDES ANALYSIS METHODS AND THEIR PRELIMINARY APPLICATION TO BARLEY PROTEINS STUDY

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Summary

N-glycosylation is the most frequently studied plant protein post-translational modification. The analysis of *N*-glycopeptides after protein proteolytic digestion offers information about the structure of both oligosaccharide and peptide moiety. However, this method has so far been less commonly used. Since glycopeptides hardly ionize during MS analysis in the presence of non-glycosylated peptides, they need to be separated from the complex peptide mixture. In this study, the glycopeptides enrichment, purification and analysis methods were successfully optimized on two standard *N*-glycoproteins. Concanavalin A (ConA) lectin tips were used for glycopeptide capturing, and obtained fractions were purified on carbon tips and analyzed using MALDI-TOF mass spectrometry. The differences in the CID fragmentation of certain types of glycopeptides were found. This technique was then applied to glycopeptide analysis of barley grain and malt proteins. Several barley glycopeptides were found, however, their identification was very difficult. More proteins separation techniques will be required before this enrichment procedure in further studies.

1. Introduction

Glycosylation, i.e. the covalent linkage of an oligosaccharide side chain to a protein, represents one of the most common protein post-translational modification and the most frequently studied modification in plant proteins. *N*-glycans contain a common trimannosyl-chitobiose core $Man_3GlcNAc_2$ and according to attached saccharide residues, they can be divided in three types: high mannose, complex and hybrid[1,2].

The direct analysis of intact glycopeptides by mass spectrometry offers sequence information on both peptide and glycan moiety. However, this method has so far been less commonly used. The analysis of glycopeptides after proteolytic digest without any pretreatment is difficult because non-glycosylated peptides interfere with ionization of glycopeptides. Therefore, the removing of non-glycosylated peptides from the proteolytic digest is necessary for efficient analysis. Lectin-affinity chromatography is often used to glycopeptides enrichment. Concanavalin A (ConA) is one of the most well characterized and widely used lectins. MALDI-TOF MS/MS analysis of *N*-glycopeptides results in specific fragment ion signals pattern of chibitose core fragmentation that differs in the case of non-core- and core-fucosylated *N*-glycopeptides[3,4].

2. Experimental

Proteins were digested (after reduction and alkylation) overnight at 37 °C using chymotrypsin or trypsin (enzyme-to-protein ratio of 1:50, w:w). Glycopeptides enrichment was performed on ConA lectin TopTips (Glygen Corporation, MD, USA) according to the manufacturer manual. Obtained fractions were purified using carbon Supel Tips and spotted on MALDI target with 2,5-dihydoxybenzoic acid (DHB) or ferulic acid matrix solution. MALDI-TOF MS experiments were performed on AB SCIEX TOF/TOF 5800 System equipped with a 1 kHz Nd:YAG laser (AB SCIEX, Framingham, MA, USA).

3. Results and discussion

3.1. Optimization of *N*-glycopeptide analysis

The analysis of *N*-glycopeptides was optimized using two standard glycopeptides, ribonuclease B (RNase B) and horseradish peroxidase (HRP), after both tryptic and chymotryptic digestion. For glycopeptide enrichment, purification and MALDI-TOF analysis, various methods and their conditions were tested. The best results were obtained by ConA affinity separation and carbon purification.

Glycopeptides were analyzed by MALDI-TOF mass spectrometry and obtained MS/MS spectra were manually interpreted according to the literature[3]. GlcNAc oxonium ions (m/z 204 and 186 and/or 168), typical for CID fragmentation of glycopeptides, and the characteristic fragment patterns for non-core-fucosylated or core-fucosylated *N*-glycopeptides were searched in MS/MS spectra. Thereby, the masses of both peptide and glycan moieties were determined. The structures of glycan and peptide moieties were identified according to fragmentation signals.

Both unbound and bound fractions from ConA standard glycopeptides enrichment were analyzed by MALDI-TOF MS and compared. Significant differences

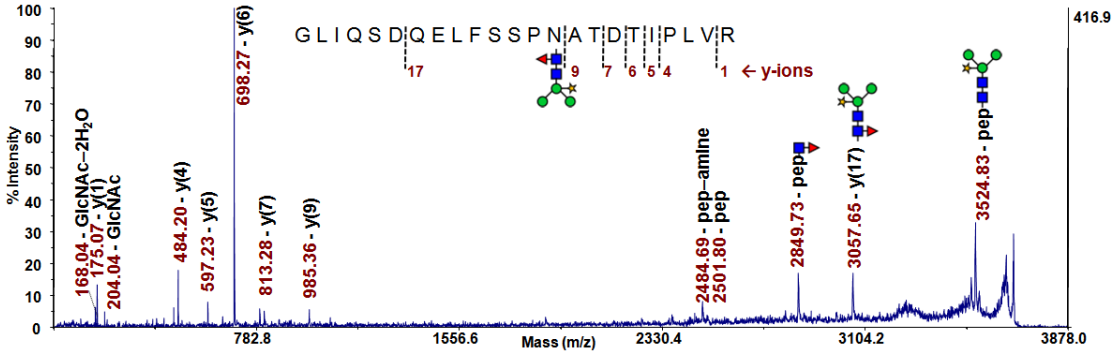

Fig. 1. **MS/MS spectrum of the tryptic glycopeptide G_{272}-R_{294} from horseradish peroxidase containing $GlcNAc_2Fuc_1Man_3Xyl_1$ *N*-glycan (precursor at m/z 3670.9). Ferulic acid was used as a matrix;** ■ GlcNAc ● mannose ★ xylose ▶ fucose

were observed and several glycopeptides were detected in the bound fraction. While RNase B contains high-mannose-type glycans, plant HRP contains various complex-type glycans. The illustrative MS/MS spectrum of one HRP glycopeptide is shown in Fig. 1.

3.2. Analysis of barley *N*-glycopeptides

Optimized methods were applied to the study of barley glycoproteins. The aqueous extracts of grain and malt digested with chymotrypsin were used for enrichment of glycopeptides. Several differences were observed between the MS spectra of the bound and unbound ConA fractions. Six potential glycopeptides were found in the grain sample and nine of them in the malt sample. The illustrative MALDI-TOF fragmentation spectrum of one barley malt glycopeptide is shown in Fig. 2. According to the fragmentation it was deduced that it contains high-mannose glycan $GlcNAc_2Man_6$. However, the fragmentation of all possible glycopeptides was not optimal and their identification was difficult. Obtained glycopeptides originate from a wide range of glycoproteins that are present in the complex grain or malt aqueous extract. Therefore, more protein separation techniques will be required before this enrichment procedure in further studies.

4. Conclusions

In this study, the enrichment of glycopeptides, subsequent glycopeptide purification and mass spectrometric analysis were successfully optimized. The principles of glycopeptides fragmentations and their interpretation were found out. Optimized methods and acquired experiences were utilized in subsequent preliminary investigation of barley grain and malt glycopeptides.

This work was supported by the Czech Science Foundation (grant No. P503/12/P395) and by institutional support RVO:68081715 of Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic, v.v.i. This project is also co-financed by the European Social Fund and the state budget of the Czech Republic (CZ.1.07/2.3.00/20.0182).

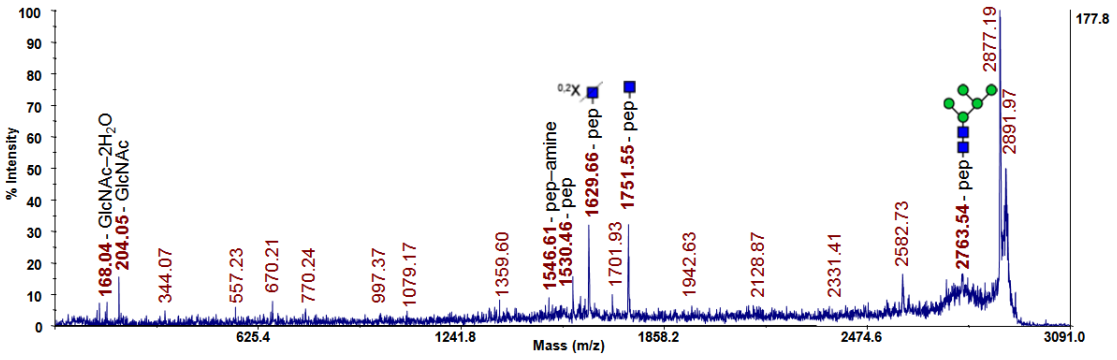

Fig. 2. **MS/MS spectrum of the chymotryptic glycopeptide from barley malt (precursor at m/z 2925). DHB was used as a matrix;**
■ GlcNAc ● mannose

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NEW APPROACH IN ELECTROCHEMICAL IMMUNOMAGNETIC BIOSENSORS FOR PROTEIN DETECTION

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Summary

The simple, sensitive, and selective method for electrochemical detection of ovalbumin is presented. This method is a combination of the selective immunocomplex formation on the surface of magnetic carriers for easy manipulation and preconcentration with a very sensitive electrochemical detection. The evaluation of antigen content is based on electrochemical detection of quantum dots as labels of secondary antibodies, which allows detecting of low amounts of target antigen.

1. Introduction

The detection of low concentrations of target antigen (i.e. proteins) is nowadays of great importance in biosensing area. New trends in this field combine selective immunochemical reaction with the quantum dots (QDs) as a label of secondary antibodies. This approach represents highly selective and sensitive detection method.

QDs are nanoscaled inorganic crystals characterized by very interesting optical and electronic properties[1]. These nanocrystals have a core-shell structure and diameter from 2 to 10 nm. The core is usually composed of elements such as Cd, Pb, or In. The shell usually consists of ZnS (ref.[2]).

Considering the composition, QDs have a great potential for electrochemical detection. Electrochemical techniques are known to have unique advantages in terms of both economic features and high sensitivity, e.g. in the determinations of low levels of heavy metals in different samples[4]. Anodic stripping voltammetry (ASV) in particular utilizes the efficient preconcentration of analyte, which can be combined with sensitive detection step using pulse techniques, like square-wave voltammetry (SWV) or differential pulse voltammetry (DPV). Such techniques effectively discriminate the faradaic current from the background current and they are often used for the identification of the redox processes and the determination of the corresponding current values[1]. Nowadays, the square-wave anodic stripping voltammetry (SWASV) is widely applied in electroanalysis of various species[3].

Additionally, there is a continuous push to use miniaturized electrochemical sensors and integrate as many electrodes as possible on one substrate. Therefore, the use of screen-printed sensors for electrochemical detection of ovalbumin in small sample volumes is presented in this contribution.

2. Experimental

2.1. Voltammetric measurements

All voltammetric measurements were performed with PalmSens interface (PalmSens, Netherlands). With regard to minimization of sample volume the detection of QDs were performed with mercury film screen-printed carbon electrode (ItalSens, Italy). Each screen-printed electrode was pretreated by applying the potential of –1.1 V for 300 s before use. Afterwards, the square wave voltammetric scans were carried out until low and stable background was obtained.

Prior to voltammetric scan, the nanometer-sized quantum dots Qdot®565 ITK Carboxyl Quantum Dots CdSe/ZnS (Life Science) were firstly dissolved with 50 µl HCl. Different concentration of HCl (0.1 M, 1 M, and 3 M) and different time (1, 3, 5, and 10 min) for QDs dissolution were tested. After optimization of QDs detection, the calibration curve of the quantum dots was ascertained using SWASV technique with 2 min of heavy metals accumulation.

2.2. Ovalbumin detection

The immunocomplex for specific immunocapture of ovalbumin was formed onto surface of SiMag-carboxyl magnetic particles (Chemicell, Germany). The anti-ovalbumin antibodies (Tetracore, USA) were covalently immobilized onto surface of magnetic particles. The secondary antibodies were prepared by conjugation of anti-ovalbumin antibodies with Qdot®565 ITK Carboxyl Quantum Dots. The evaluation of ovalbumin concentration was than based on electrochemical detection of QDs presence in analysed sample. Finally, the calibration curve for ovalbumin detection was ascertained.

3. Results and discussion

In this contribution, the electrochemical immunomagnetic assay of ovalbumin with subsequent

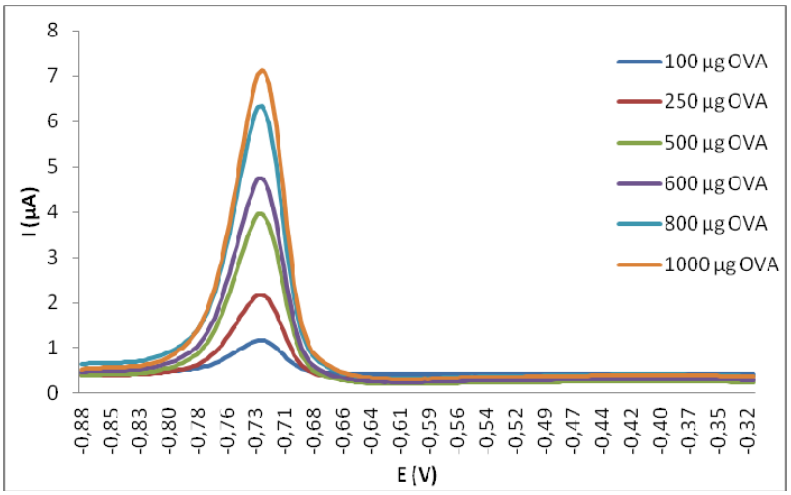

Fig. 1. **SWASV voltammograms of calibration in electrochemical immunomagnetic assay for ovalbumin – system anti-ovalbumin–ovalbumin–anti-ovalbumin labeled with QDs Q565 in 0.1 M HCl for 2 min heavy metals acumulation**

square-wave anodic stripping voltammetry of quantum dots is presented. This technique takes advantage of that quantum dots are composed of heavy metals such as cadmium and lead, which are easily electrochemically detected.

On the basis of optimal detection conditions, this technique was also used for the evaluation of the conjugation efficiency of secondary antibodies with QDs. This conjugate was subsequently used as secondary antibody for determination of the presence of ovalbumin as model system in the sample. The trend of increasing electrochemical response with increasing concentration of quantum dots and therefore ovalbumin is evident.

The use of magnetic particles of nanometer size enables highly efficient separations of target biomolecules due to their large surface area for specific ligand immobilization. They also allow to preconcentrate and separate target ligands on the surface of transducer with an aid of magnetic field.

4. Conclusions

Nowadays, the quantum dots have many interesting optical features for biosensing applications and have emerged not only in optical sensing strategies, but also in electrochemical sensing approaches. Electrochemical immunomagnetic sensor for detection of ovalbumin as a model protein, which is based on QDs determination, is presented. Such biosensor is attractive due to good availability of screen-printed electrodes, simple use, fast analysis, low detection limits and possibility of miniaturization and can be adopted for another protein detection with slight modification only.

This work was financially supported by the Czech Science Foundation P206/12/0381 and 7. FP EU project „NaDiNe“ No 246513.

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NEW SENSOR FOR DNA MUTATION DETECTION

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Summary

We present a design and synthesis of a new sensor intended for the genomic analysis in molecular cancer research. This sensor is based on the Förster resonance energy transfer (FRET) and can be used for the detection of complementary oligonucletide chains based on probe hybridization. Quantum dots (QDs) with their unique optical properties serve as suitable donors of energy in FRET.

1. Introduction

The process of FRET is a photophysical phenomenon through which an energy absorbed by a fluorophore (the energy donor) is transferred nonradiatively by dipole-dipole interactions to the second fluorophore (the energy acceptor)[1]. The efficiency of energy transfer between the donor and acceptor is sensitive to their distance (1–10 nm). The main advantages of QDs, when compared with the conventional organic fluorescent dyes, include absence of photobleaching, wide range of excitation wavelengths and narrow emission the wavelength of which can be modulated by the QD size[2]. Moreover, their high extinction coefficients are prerequisites for absorption of a high amount of energy. The energy is transferred effectively from donor to acceptor and emitted at a longer wavelength.

2. Experimental

We focused on the synthesis of CdTe QDs of a size of 3.2 nm with a maximum emission wavelength of 570 nm passivated by inorganic salts (CdS, ZnS). Mercaptopropionic acid (thiolated ligand) was covalently bonded to the surface to make QDs water soluble. The CdTe QDs were conjugated with a specific oligonucleotide sequence *via* zero-length cross-linkers[3] to form a luminescent probe (donor of FRET). ROX-labelled (6-carboxyrhodamine) PCR fragment from the studied sample serves as an acceptor. The sample-probe hybridization was performed using a standard annealing protocol and followed by fluorescence measurement.

3. Results and discussion

Capillary Electrophoresis with Laser Induced Fluorescence Detection (CE-LIF) was used for analyses of products of conjugation reaction and hybridization reaction. Products of conjugation reaction between QDs and aminated oligonucleotide in ratio 1:1 provided single peak. Thus the absence of peaks of free reactants (QDs) indicates complete conversion.

Hybridization experiments were done in parallel with ROX-labeled complementary and non-complementary oligonucleotides. Each of the oligonucleotides showed a single peak at a 1:1 ratio. By increasing the amount of complementary oligonucleotides a secondary peak was observed. In case of noncomplementary hybridization only single peak remained in various ratios of the reactants. The result indicates nonspecific interaction of both hybrides. After the hybridization experiment with complementary oligonucleotide, an increase of the fluorescence emission spectra at the wavelength of 610 nm confirmed the function of FRET.

4. Conclusions

We present the design, physico-chemical properties and results of the testing of a sensor based on a conjugate of QD and oligonucleotide probe. Hybridization reaction with complementary and non-complementary oligonucleotides resulted in different separation profiles. An increase in fluorescence signal of the hybridized product at the ROX emission wavelength indicates transfer of energy.

This work was supported by Technology Agency of the Czech Republic (TA02010672) and by institutional support RVO 68081715 of Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic, v.v.i. This project is co-financed by the European Social Fund and the state budget of the Czech Republic (CZ.1.07/2.3.00/20.0182).

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PIEZOELECTRIC BIOSENSOR COUPLED TO CYCLONE AIR SAMPLER FOR DETECTION OF MICROORGANISMS

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Summary

Even though microorganisms are common part of our lives, some of them are dangerous and must be monitored. The traditional microbiological methods are reliable but slow. In some cases, namely in military, key requirement is fast detection which can be provided by biosensors. In this work, the piezoelectric biosensors providing fast, specific and cheap way of detection are described. Three strains of *E. coli* (BL21, DH5α and K-12) were chosen as safe model microbes and the detection using the developed piezoelectric biosensor was carried out (LOD 10^6 CFU mL^{-1}, total analysis time 20 min). Bioaerosol chamber was constructed to allow safe dissemination of microbes in air and experiments with bioaerosol. The generated bioaerosol was sampled using the cyclone air sampler and analyzed with the on-line coupled piezoelectric biosensor. The levels of 10^6 CFU L^{-1} of *E. coli* in air were successfully detected in less than 26 min which demonstrates possible application of this system for rapid screening of microorganisms in the air.

1. Introduction

Detection of microorganisms by the traditional microbiological procedures is reliable but usually lengthy. In the last years, fast and sensitive methods of detection based on biosensors are being developed. The good specificity of biosensors allows analysis of complex matrices of samples from military and public protection, clinical and food control applications[1].

The importance of detection of microorganisms spread in the air arises with the risk of terroristic attacks. Most of the methods currently used are based on surface-enhanced Raman spectrometry[2] or electrochemical biosensors[3], but new techniques are still being developed[4]. Piezoelectric biosensors are based on changes of the resonant frequency of piezoelectric crystal. This frequency change depends on the amount of mass bound to the sensor surface. Piezoelectric biosensors are sensitive, cheap and easy to use and therefore suitable for label-free detection of microorganisms[5].

2. Experimental

2.1. Microorganisms and antibodies

Escherichia coli was chosen as a safe model microorganism because of its fast and easy cultivation and good availability of commercial antibodies. Three strains (BL21, DH5α and K-12) were tested. Cultivation was done both aerobically and anaerobically using LB Broth (Duchefa Biochemie, Netherlands) over night at 37 °C. The obtained suspension was centrifuged twice at 4500 RCF for 15 min. Each time, the supernatant was discarded and the pellet was resuspended in 50 mM phosphate buffered saline pH 7.4 (PBS). Concentration of microorganisms was determined using the McFarland scale.

Antibody Abcam ab25823 was used for detection of strains BL21 and DH5α. In case of the strain K-12 antibody Serotec 4329-4906 was chosen.

2.2. Piezoelectric immunoassay

To prepare piezoelectric biosensor, gold electrodes of 10 MHz quartz crystals (ICM, USA) were cleaned 30 min in acetone and then incubated in cysteamine (20 mg mL^{-1} in water, 2 hours) to form a self-assembled monolayer. The 1 hour incubation in 5% glutaraldehyde in PBS followed. One type of sensors was subsequently modified for 20 hours by 1 mg mL^{-1} solution of staphylococcal protein A (SpA) which specifically binds the Fc fragment of antibodies. For the other type, antibody was bound directly covalently (100 μg mL^{-1}, 20 hours at 4 °C) after activation with GA. Finally, free reactive aldehyde groups were deactivated using 50 mM ethanolamine for 30 min.

The prepared immunosensor was placed in a flow-through cell and affinity interactions were measured in real-time using QCM Analyzer (Keva, Czech Republic). All measurements were performed in PBS with a flow rate 25 μL min^{-1}. Samples were flown over the sensor for 10 min followed by 10 min dissociation time. Regeneration was done using 50 mM NaOH.

2.3. Bioaerosol chamber

Bioaerosol chamber was a hermetically closed box (volume 1 m^3) made of plexiglass placed on a movable table to allow easy transportation. Air coming in and leaving out of the chamber was filtered by HEPA filters (AirFilters, Czech Republic) connected to air pumps (Hurricane, Italy) that allow fast exchange of air. To measure physical conditions inside the chamber, sensor system Comet 7511 (Comet, Czech Republic) was used.

Humidity was regulated with the help of the humidifier Super Fog (LuckyReptile, Germany).

Aerosol was generated by a vibrating ultrasonic piezoelectric actuator placed in a beaker, where the liquid sample with microbes was injected. Thus formed aerosol was spread using three 12-cm fans (Nexus, Netherlands). Level of particles was measured by the counter Met One 3400 (Hach, USA). The air was gathered by a cyclone air sampler SASS 2300 (Research International, USA) which was connected to the piezoelectric immunosensor. The whole system was controlled remotely by a computer connected through LAN network using router and serial/ethernet adapters.

2.4. Measurement of bioaerosol

Before measurements, the bioaerosol chamber was disinfected using ethanol, closed and air in the chamber was filtered (30 min in/out circulation) to decrease the number of particles inside. Relative humidity was increased to 80 %. Dissemination was done by the piezoelectric actuator; to prevent damage of microbes, 5 shorter cycles (per 60 s with 30 s break) were performed instead of one longer dissemination. During this procedure 1 mL of bacterial suspension (10^9 CFU mL^{-1}) was disseminated giving total concentration in the chamber 10^6 CFU L^{-1}. When dissemination was finished, cyclone was run for 5 min and liquid was captured in a reservoir linked to the piezoelectric biosensor. Piezoelectric immunoassay was done in the same way as mentioned in chapter 2.2.

3. Results and discussion

Interactions of *E. coli* with antibodies were studied using piezoelectric biosensors. Fig. 1 shows the interactions between various concentrations of *E. coli* DH5α and antibody Abcam ab25823 immobilized directly using GA. Limit of detection was 10^6 CFU mL^{-1} and in case of concentration 10^8 CFU mL^{-1} the signal change was 26.6 Hz. Dependence of signal change on concentration of microbe exhibited saturation character. No differences between aerobically and anaerobically cultivated microbes were observed.

Then interactions of the same type of sensor with the strain BL21 were studied, too. The sensitivity was lower than in case of DH5α (signal change 14.4 Hz for 10^8 CFU mL^{-1}) but the same LOD was achieved. Sensor with antibody bound using SpA provided higher changes of resonant frequency for high concentrations (37 Hz for 10^8 CFU mL^{-1}) but LOD was worse ($5 \cdot 10^6$ CFU mL^{-1}). When studying interactions between the strain K-12 and antibody Serotec 4329-4906 analogous results were obtained.

Measurements in bioaerosol chamber have shown that detection down to 10^6 CFU L^{-1} of air is feasible. Results for two experiments with the strain DH5α and one experiment with BL21 can be seen in Fig. 2. In agreement with previous results, DH5α gave approximately twice higher signal than BL21. Comparison of two measurements with DH5α confirms good reproducibility of the method. Similar results were achieved also in case of antibody Serotec 4329-4906 and K-12.

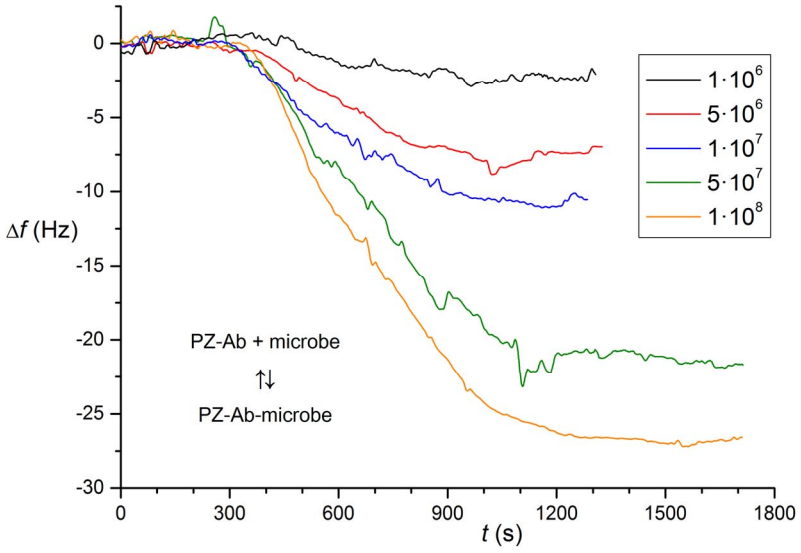

Fig. 1. **The interactions between *E. coli* DH5α and antibody Abcam ab25823 immobilized directly through GA studied using piezoelectric (PZ) immunosensor.** Change of resonant frequency (Δ*f*) in time is shown. Microbe levels are expressed as CFU mL^{-1}

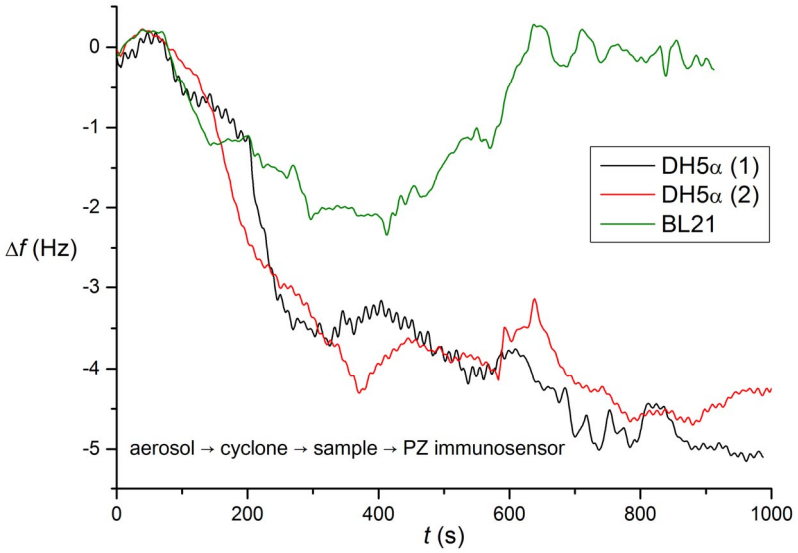

Fig. 2. **The interactions between *E. coli* strains DH5α and BL21 captured from aerosol with antibody Abcam ab25823 immobilized directly through GA.** Change of resonant frequency (Δf) in time is shown. The aerosol contained 10^6 CFU L^{-1} of *E. coli* cells

4. Conclusions

Piezoelectric biosensor for rapid detection of *Escherichia coli* was developed with a limit of detection 10^6 CFU mL^{-1} and total analysis time 20 min. Bioaerosol chamber was constructed to allow safe measurements with aerosolized microorganisms. The aerosol was gathered by a cyclone air sampler and analyzed using piezoelectric biosensor. The level of 10^6 CFU L^{-1} of *E. coli* in aerosol was successfully detected with time for cyclone capturing, sampling and QCM detection less than 26 min making this system a good alternative to other methods for detection of microorganisms in air.

The work has been supported by the Ministry of Defence of Czech Republic (projects no. OVVTUO2008001 and OSVTUO2006003) and by CEITEC – Central European Institute of Technology (CZ.1.05/1.1.00/02.0068) from European Regional Development Fund.

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COMPARISON OF CHIRAL STATIONARY PHASES BASED ON IMMOBILIZED POLYSACCHARIDES IN REVERSED PHASE MODE

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Summary

Immobilized polysaccharide-derived chiral stationary phases (CSPs) are a new type of chromatographic columns, which allow using much broader range of solvents. However, these columns are mostly used in normal phase HPLC mode. In this study we worked in reverse phase mode and we used three CSPs that differed in the type of derivatization group or in the nature of the glycosidic linkage of the polysaccharide derivatives. The columns CHIRALPAK IA, CHIRALPAK IB and CHIRALPAK IC are based on tris-(3,5-dimethylphenylcarbamate) of amylose, tris-(3,5-dimethylphenylcarbamate) of cellulose and tris-(3,5-dichlorophenylcarbamate) of cellulose, respectively.

1. Introduction

Chirality is an unique phenomena affecting human life in many aspects. Molecules that are not identical to their mirror images are kinds of stereoisomers called enantiomers. Enantiomers have identical physical properties but could differ in biological activity. Ignorance of pharmaceutical and toxicological differences of the individual enantiomer forms can cause disastrous consequences. As an example can serve chiral compound called thalidomide which was discovered as a sedative to help pregnant women with affects of morning sickness. While *R*-enantiomer had required pharmacological effect, the *S*-enantiomer caused fetal malformations.

High performance liquid chromatography (HPLC) has become a powerful technique for the development of enantioselective separations of chiral drugs and has a significant impact for pharmaceutical, food and agrochemical industries. Nowadays, chiral stationary phases (CSPs) based on polysaccharides (amylose, cellulose) have proven to be one of the most useful tools for separation of a wide range of chiral compounds. Immobilized polysaccharide-derived CSPs are a new type of chromatographic columns, which demonstrate better performance in the areas of enantioselectivity, efficiency and CSP-solvent compatibility[1–3].

2. Experimental

The tested columns CHIRALPAK IA, CHIRALPAK IB and CHIRALPAK IC are based on tris-(3,5-dimethylphenylcarbamate) of amylose, tris-(3,5-dimethylphenylcarbamate) of cellulose and tris-(3,5-dichlorophenylcarbamate) of cellulose, respectively, Fig. 1. The set of diverse chiral compounds including acidic, neutral and basic ones was tested to characterize and understand the principles of chiral recognition. This facilitates the development of new separation methods and accelerate their optimization. In the frame of the separation procedure the various types of mobile phases were tested. The acidic mobile phases were used for acidic analytes to suppress the ionization because charged analytes cannot

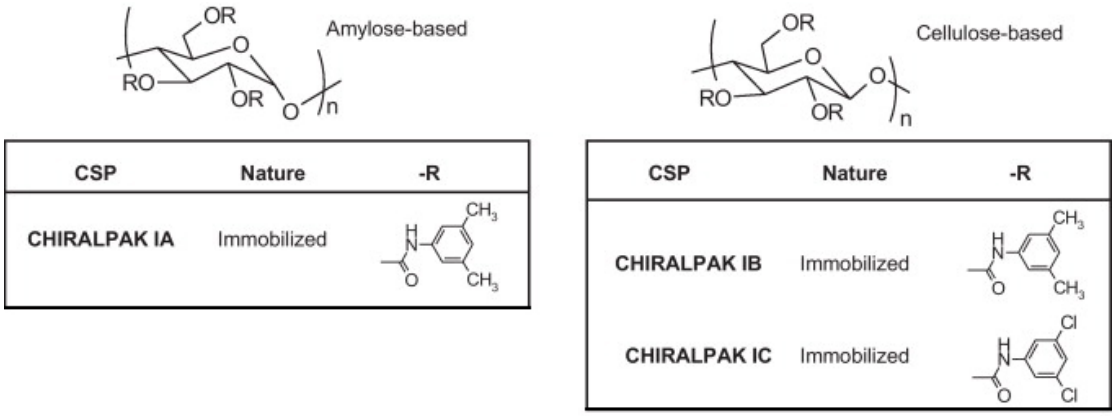

Fig. 1. **Structures of the chiral selectors**[5]

interact efficiently with CSPs (ref.[4]). Basic analytes were separated in suitable basic buffer systems or in the acidic mobile phases with considerable amount of chaotropic reagent (KPF_6, $NaClO_4$) which forms an ion pair with the positively charged analyte.

3. Results and discussion

RP mode mobile phases composed of ACN or MeOH as organic modifiers and suitable buffer systems was chosen for the enantioseparation of selected analytes. The influences of the type and the amount of organic modifier (acetonitrile, methanol) on chromatographic parameters were evaluated. The retention times of the analytes decrease with increasing the amount of organic eluent. Additionally, the same amount of acetonitrile gives a shorter retention than an equivalent amount of methanol. The effect of column temperature on enantioselectivity and resolution of the enantiomers was also studied. The chromatographic parameters of different pharmaceuticals obtained in reversed phase separation systems on three different CSPs were compared. For illustration see Fig. 2 and Fig. 3.

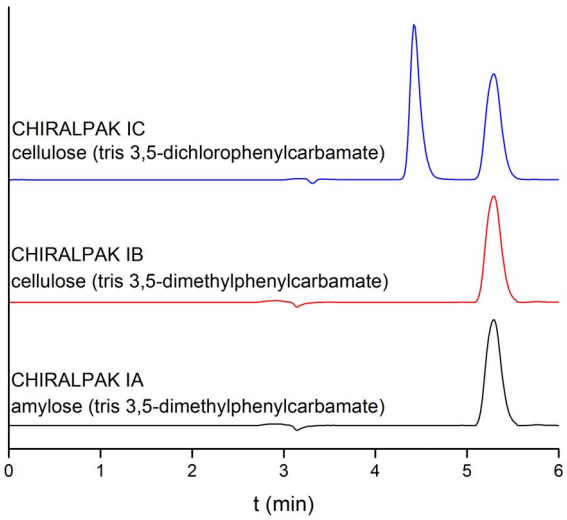

Fig. 2. **Comparison of enantioseparation of fenoprofen on three different CSPs.** Mobile phase composition: ACN/aqueous solution of formic acid, pH 2.10, 60/40 (*v/v*); temperature 25 °C; detection 254 nm

4. Conclusions

The combination of three immobilized CSPs constitutes a powerful column set for resolution enantiomers in reverse phase mode. The reversed phase

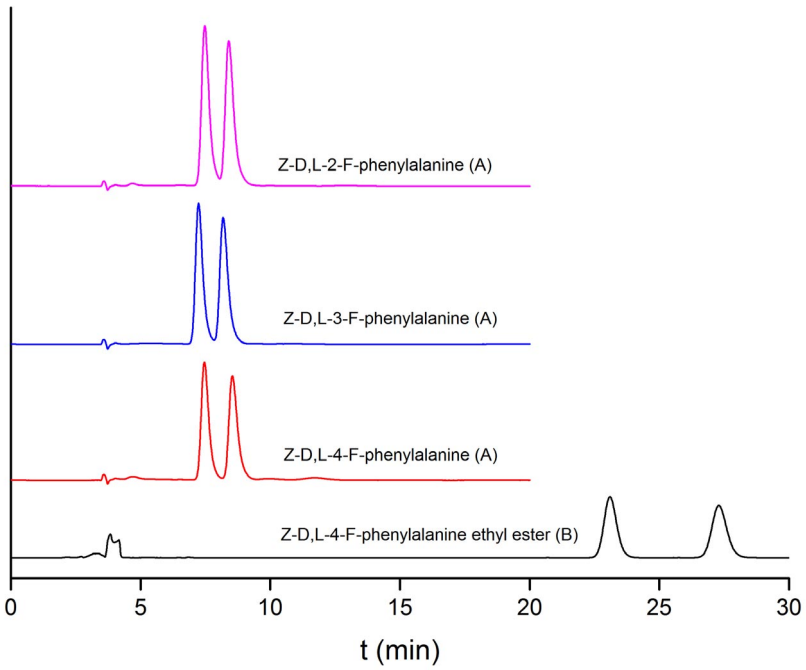

Fig. 3. **Chromatograms of enantioseparation of *N*-blocked analytes on Chiralpak IC column.** A) Mobile phase composition: MeOH/aqueous solution of formic acid, pH 2.20, 70/30 (*v/v*); B) Mobile phase composition: ACN/water 45/55 (*v/v*); temperature 25 °C; detection 254 nm

mode was proved to be suitable for separation of the majority of tested compounds. The results showed that both the polysaccharide type and substituent affect the separation behavior.

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AUTOMATED N-GLYCOSYLATION ANALYSIS FOR TRANSLATIONAL GLYCOMICS: QUO VADIS?

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Summary

The glycome is the entire set of sugars in a cell, tissue or organism at a certain time, including free and complex forms. Unlike genomes, the glycome is highly dynamic, due to the interplay of those more than 600 enzymes that can be involved in the complex pathways of protein glycosylation. In addition, glycosylation is cell, protein and site specific and epigenetic factors regulating the expression of glycosyltransferases and glycosidases may represent further diversifying mechanisms, influencing the functions of the proteins to which carbohydrates are attached. In the biomedical field, altered glycosylation is frequently associated with pathological conditions. Glycosylation modulation in the biotechnology industry, usually due to changes in bioprocessing / cell culture conditions, may alter the affectivity of biotherapeutics. In both instances, information about specific glycosylation motifs such as the type and number of sugar monomers along with their position and linkage specificity are of high importance. Glycomics, as a subset of glycobiology, systematically studies all glycan structures in a given sample. To fulfill the need of such global glycomics studies, automated, high throughput (preferably in 96 well plate formats) and robust bioanalytical platforms are required to address sample preparation (glycoprotein capture, glycan release and carbohydrate labeling), separation (capillary electrophoresis or liquid chromatography) and data processing (glycoinformatics) issues. This talk will confer the state of the art of analytical glycomics and discuss recent efforts and future prospective of this emerging field in regards to system integration, translational options and their implications in the biomedical and biopharmaceutical arena.

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FAST DETERMINATION OF CATIONS AND ANIONS ON ELECTROPHORETIC MICROCHIP IN CEREBROSPINAL FLUID

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Summary

A new analytical method for fast and direct determination of inorganic cations and anions in cerebrospinal fluid by microchip electrophoresis with conductivity detection has been proposed. Samples of cerebrospinal fluid were only diluted appropriately before the analysis. Two different electrolytes were used, one for cationic, and another for anionic separations, at pH 3.1 and 4.3, respectively. Limits of detection were in the range 0.015–0.046 mg L^{-1} and 0.062–0.218 mg L^{-1} for cations and anions, respectively. Repeatabilities of migration times for both cations and anions were up to 1.6 %. Repeatabilities of peak areas for cations were between 2.4 and 4.5 % and for anions between 0.9 and 3.9 %. Recoveries of studied cations and anions in tested samples ranged from 92 to 106 %.

1. Introduction

Cerebrospinal fluid (CSF) analysis is mainly used in diagnostics of central nervous system diseases[1]. It is known that increase or decrease of certain inorganic cations and anions in the CSF can indicate several illnesses, e.g. Alzheimer's disease[2], multiple sclerosis[3], meningitis[4] and Parkinson's disease[5].

Since CSF is a complex matrix the analytical methods with good selectivity and sensitivity are to be used. In addition, in order to achieve fast analysis with minimum sample consumption miniaturized analytical techniques are being used. Microchip capillary electrophoresis fulfills all of these demands.

The simultaneous analysis of cations and anions in complex biological samples is very rare, due to their different concentration levels in such samples. Therefore, microchip electrophoresis methods for separations of inorganic cations (ammonium, calcium, magnesium, sodium and potassium) and anions (chloride, sulfate, nitrite and nitrate) using two background electrolytes (BGEs) on the same microchip were proposed.

2. Experimental

2.1. Instrumentation

Separations were carried out on PMMA column coupling (CC) microchip with integrated conductivity sensors (IonChipTM 3.0, Merck, Darmstadt, Germany). MicroCE analyzer consisted of electrolyte and electronic unit. Main components of an electrolyte unit were peristaltic micropumps and membrane driving electrodes. Peristaltic micropumps were used to transport BGE and sample solutions to the microchip. The membrane driving electrodes were used to suppress disturbances due to the bubble formation during the separation run. An electronic unit delivered the stabilized driving current to the counter-electrode, drove the peristaltic micropumps and interfaced the microCE analyzer to a PC. This unit also included the measuring electronics of the contact conductivity detectors. Monitoring of the analysis as well as collecting the data from conductivity detectors and their evaluation were done using MicroCE Win software, version 2.4 (Merck).

2.2. Chemicals, electrolytes and samples

Chemicals used for the preparation of electrolyte solutions were obtained from Sigma-Aldrich (Steinheim, Germany). Model samples were prepared from chemicals of p.a. purity (Sigma-Aldrich).

CSF samples were collected from three patients with symptoms of neurodegenerative diseases in the 1st Neurological Clinic on Faculty of Medicine, Comenius University in Bratislava and stored at –40 °C in 1.5 mL polypropylene microcentrifuge tubes (VWR, Wien, Austria). Prior to the analysis samples were defrosted, homogenized and after appropriate dilution analyzed.

3. Results and discussion

3.1. Analysis of model samples

Capillary zone electrophoresis (CZE) separations in two different BGEs were performed with model samples during one day on the same microchip. Repeatabilities of migration time and peak area were evaluated from four repeated CZE analyses at three different concentration levels. RSD values of migration times of cations were within 1.2 % and of anions within 0.4 %. RSD values of peak area ranged from 0.3 to 5.6 % for cations and from 0.6 to 5.9 % for anions.

Standard procedure was used for estimation of limit of detection (LOD) based on three time of signal to noise

ratio (3×S/N). LOD was calculated for each of the cations and anions. LOD of cations ranged from 0.015 to 0.046 mg L^{-1} while LOD of anions were from 0.062 to 0.218 mg L^{-1}.

3.2. Analysis of real samples

Electropherograms obtained from analyses of both cations and anions are shown in Fig. 1. Samples were diluted 1500–2500 times for analysis of cations. They were directly analyzed using CZE approach, since under these dilution factors all cations were separated with good resolution.

CZE-CZE approach had to be used in order to determinate anionic micro-constituents since there is excess of chloride in CSF. Initially, CZE separation was performed in the first channel of the microchip and chloride, the most mobile analyte and, at the same time, macro-constituent, was removed into the bifurcation region. Subsequently, by switching the direction of the driving current from the first to the second channel, the rest of chloride and all other analytes migrating behind, were transferred to the second channel, where CZE separation was carried out. Fig. 1 shows that chloride was not completely removed in the first channel which can be ascribed to diffusion driven transfers of chloride from the bifurcation region to the second separation channel.

Three different CSF samples were analyzed on the same microchip. Repeatabilities of migration time and peak area were evaluated from four repeated CZE analyses. RSD values of migration times for cations were from 0.2 to 1.6 % and for anions ranged from 0.2 to 1.0 %. RSD values of peak areas ranged from 2.4 to 4.5 % for cations and for anions were from 0.9 to 3.9 %. Repeatabilities of both migration times and peak areas in CSF samples were similar to those in model samples.

Concentrations of cations and anions obtained from the analyses of CSF samples corresponded to a large extent with concentrations of studied ions found in literature. Recoveries of the cations and anions in CSF samples ranged from 92 to 106 %.

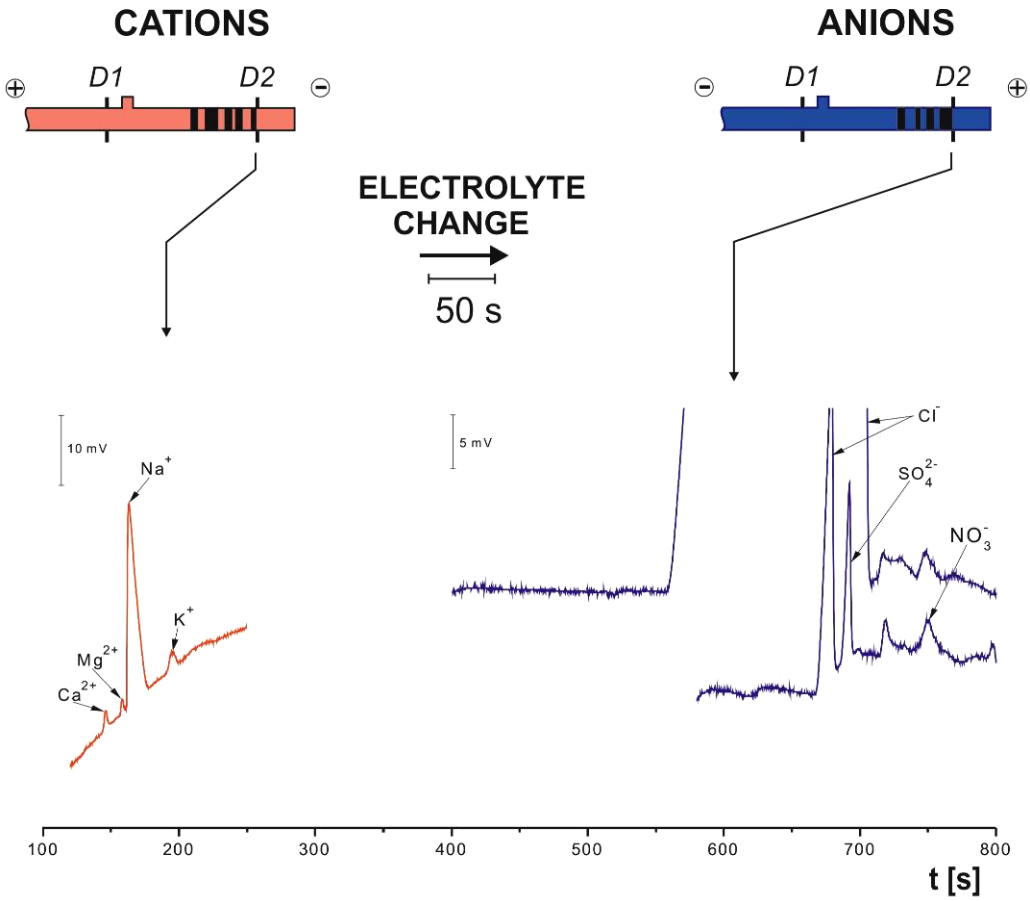

Fig. 1. **CZE separations of inorganic cations and anions present in CSF sample.** Separations were carried out in cationic and anionic BGE at pH 3.1 and 4.3, respectively. Driving current was stabilized at 50 μA for separation of cations and 30 μA for separation of anions in both separation channels

4. Conclusions

This work dealt with separation of cations and anions by CZE in three CSF samples on CC microchip. CSF samples with suspected neurological diseases were analyzed without any pretreatment other than dilution. Given high concentration of chloride in CSF, CZE-CZE approach has been used for determination of anionic micro-constituents, sulfate and nitrate. CZE approach was used for determination of chloride. For determination of cations CZE approach was used.

The employed methods are suitable for fast and sensitive determinations of the studied cations and anions (LOD in the range 0.02–0.05 and 0.06–0.22 mg L^{-1} for cations and anions, respectively) in CSF without any pretreatment.

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