

CECE 2016

13th International Interdisciplinary
Meeting on Bioanalysis

“... bringing people
and ideas together ...”

October 17 - 19, 2016
Hotel Continental
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Institute of Analytical Chemistry of the CAS, v. v. i., Brno, Czech Republic

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Find the meeting history and more at www.ce-ce.org

Foreword

Welcome to CECE 2016, the 13th International Interdisciplinary Meeting on Bioanalysis. As in the previous years, it is our goal to: “bring together scientists from different disciplines who may not meet at other meetings”. CECE Junior will follow two days of invited speaker’s lectures and poster sessions will be open during all three days. The organizers want to thank all invited speakers, sponsors and participants for their continuing support. Please, check our web at www.ce-ce.org for more information about the history, programs, photos and videos from the previous years.



Brno, October 8, 2016

The Medal of Jaroslav Janák

The Medal of Jaroslav Janák for contributions to the development of analytical sciences was established by the Institute of Analytical Chemistry. Named after the inventor of the gas chromatograph (patented in 1952), founder of the institute (1956) and its long-term director, the medal is awarded to scientists who have significantly contributed to the development of separation sciences.



In 2016, the Medal of Jaroslav Janák goes to **Prof. Petr Boček**. In the early 1980' with the fresh university diploma, I started working in the group of electromigration methods headed by Petr Boček, a smiling friendly man commanding respect not only as a former sport wrestler but also as someone who knew practically everything about analytical separations and, especially, electrophoresis. There were fancy looking plexiglass instruments (often called chips in today's papers) powered by sparking high voltages. It was capillary isotachopheresis (ITP) and Petr's group was one of the world top laboratories in ITP. While ITP was the king of capillary separations in the early 1980's Petr was already interested in capillary zone electrophoresis and presented some of the early results at the Electrophoresis Forum conferences organized by Prof. B. J. Radola in Munich, Germany. That time Prof. Radola also founded the journal Electrophoresis and invited Petr to become a member of the editorial board soon becoming an Associate Editor (1992-2000) and since 2001 serves as the Senior Deputy Editor. As an author and co-author of over 250 scientific papers and monographs on Chromatography and Electrophoresis, Petr had a major impact on the development of analytical separations and with over 7000 citations belongs to the most cited analytical



chemists not only in the Czech Republic. He is regarded as one of the founders of the "Czech capillary electrophoresis school" highly prized especially for explanation and exact description of many electromigration related phenomena. Besides heading his department Petr was also the director of the Institute of Analytical Chemistry (1993-2001). It was my pleasure and privilege to know and work with Peter for many years. I wish to congratulate him to all his achievements and thank for all the work he has done for the Institute of Analytical Chemistry and science in general.

Franta Foret

Program of the CECE 2016

Hotel Continental, Brno, Czech Republic, October 17-19, 2016

8:00 – 15:00 **Registration (Monday, Tuesday)**

Monday, October 17

9:00 – 9:15 **CECE 2016 - Opening remarks**

9:15 – 9:45

Jana Krenkova

Institute of Analytical Chemistry of the CAS, v. v. i., Brno, Czech Republic

A NOVEL NANOSPRAY LIQUID JUNCTION INTERFACE FOR VERSATILE CE-MS

9:45 – 10:15

Erdmann Rapp

Max Planck Institute for Dynamics of Complex Technical Systems, Magdeburg, Germany

XCGE-LIF: A POWERFUL TOOL IN THE GLYCOANALYTICAL TOOLBOX

10:15 – 10:45

Coffee break

10:45 – 11:15

Guillaume Laurent Erny

University of Porto, Porto, Portugal

UNLEASHING UNTARGETED HYPHENATED MS ANALYSIS WITH CHEMOMETRICS

11:15 – 11:45

Andrew Ewing

Chalmers Univ. and Univ. of Gothenburg, Göteborg, Sweden

ANALYTICAL MEASUREMENTS WITH CAPILLARY ELECTROPHORESIS, FROM ELECTROCHEMICAL CYTOMETRY TO ALZHEIMER'S PEPTIDE IN CSF

11:45 – 12:15

Andras Guttman

University of Veszprem, Veszprem, Hungary

GLYCOHISTOPATHOLOGY: MINING THE FFPE DEPOSITORIES BY CAPILLARY ELECTROPHORESIS

12:15 – 14:15

Lunch break – poster session

- 14:15 – 14:45 **Steven Wilson**
University of Oslo, Oslo, Norway
 OPEN TUBULAR LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY: A POWERFUL AND VERSATILE TOOL FOR BIOANALYSIS
- 14:45 – 15:15 **Radim Chmelik**
Brno University of Technology, Brno, Czech Republic
 LIVE-CELL QUANTITATIVE PHASE IMAGING BY COHERENCE-CONTROLLED HOLOGRAPHIC MICROSCOPY
- 15:15 – 15:45 **Kareem Elsayad**
Vienna Biocenter Core Facilities, Vienna, Austria
 UNRAVELLING AND UNDERSTANDING THE MECHANICAL PROPERTIES OF PLANTS USING BRILLOUIN LIGHT SCATTERING MICROSPECTROSCOPY
- 16:15 – 18:00 **City walk with invited speakers**

Tuesday, October 18

- 09:30 – 10:00 **Stavros Stavrakis**
ETH Zurich, Zurich, Switzerland
 DROPLET-BASED MICROFLUIDICS: HIGH-THROUGHPUT EXPERIMENTATION ONE DROP AT A TIME
- 10:00 – 10:30 **Peter Ertl**
Vienna University of Technology, Vienna, Austria
 ORGAN-ON-CHIP TECHNOLOGY: MONITORING DYNAMIC CELL RESPONSES UNDER CONTROLLED PHYSIOLOGICAL CONDITIONS
- 10:30 – 11:00 **Coffee break**
- 11:00 – 11:30 **Blanca Lapizco-Encinas**
Rochester Institute of Technology, Rochester, USA
 PARTICLE CAPTURE AND ENRICHMENT EMPLOYING NON-UNIFORM ELECTRIC FIELDS AND INSULATING STRUCTURES

- 11:30 – 12:00 **Zdeněk Hurák**
Czech Technical University, Prague, Czech Republic
SINGLE-PARTICLE HIGH-PRECISION MICRO-
MANIPULATION USING DIELECTROPHORESIS
- 12:00 – 14:00 **Lunch break – poster session**
- 14:00 – 14:30 **Daniel Georgiev**
University of West Bohemia, Pilsen, Czech Republic
MANAGING CELL HETEROGENEITY IN MICROFLUIDIC
DESIGN
- 14:30 – 15:00 **Michael V. Gorshkov**
Russian Academy of Sciences, Moscow, Russia
PROTEOGENOMICS AND SOME OF THE PITFALLS OF
THE PROTEOMIC SIDE OF THIS COIN
- 15:00 – 15:30 **Jana Roithova**
Charles University, Prague, Czech Republic
HELIUM TAGGING INFRARED PHOTODISSOCIATION
SPECTROSCOPY
- 15:30 – 16:00 **Renato Zenobi**
ETH Zurich, Zurich, Switzerland
ON-LINE ANALYSIS OF EXHALED BREATH VIA
SECONDARY ELECTROSPRAY IONIZATION MASS
SPECTROMETRY
- 16:00 – 16:05 **Invitation to CECE 2017, Veszprém, Hungary**
- 19:00 **Conference dinner with the traditional Moravian music**

Wednesday, October 19

- 9:00 – 9:15 **CECE Junior opening**
- 9:15 – 9:30 **Jasna Hradski**
Department of Analytical Chemistry, Faculty of Natural Sciences, Comenius University in Bratislava, Bratislava, Slovak Republic
DETERMINATION OF COUNTERION IN CANCER TREATMENT DRUG BY MICROCHIP ELECTROPHORESIS
- 9:30 – 9:45 **Renata Gerhardt**
Leipzig University, Leipzig, Germany
ON-CHIP HPLC WITH INTEGRATED DROPLET MICROFLUIDICS FOR FURTHER DOWNSTREAM PROCESSES
- 9:45 – 10:00 **Paweł Pomastowski**
Department of Environmental Chemistry and Bioanalytics, Faculty of Chemistry, Nicolaus Copernicus University, Torun, Poland; Interdisciplinary Centre of Modern Technology, Nicolaus Copernicus University, Torun, Poland
THE MALDI IONIZATION AS TOOLS OF MODERN BIOANALYTICS
- 10:00 – 10:15 **Lenka Portychová**
Department of Analytical Chemistry, Palacký University, Olomouc, Czech Republic; Research Institute for Organic Synthesis, Inc., Rybitví, Czech Republic
DETERMINATION OF URINARY INDICAN AND CREATININE USING AN UHPLC/ECD-DAD METHOD
- 10:15 – 10:30 **Viorica Railean-Plugaru**
Department of Environmental Chemistry and Bioanalytics, Faculty of Chemistry, Interdisciplinary Centre for Modern Technologies, Nicolaus Copernicus University, Toruń, Poland
BIO SILVER NANOPARTICLES AS A NEW OPPORTUNITY TO MEDICAL TREATMENT
- 10:30 – 11:00 **Coffee break**

- 11:00 – 11:15 **Marton Szigeti**
MTA-PE Translational Glycomics Group, University of Pannonia, Veszprem, Hungary; Horvath Csaba Memorial Institute of Bioanalytical Research, University of Debrecen, Hungary
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Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic
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- 11:30 – 11:45 **Adriana Arigò**
Department of “Scienze Chimiche, Biologiche, Farmaceutiche ed Ambientali”, University of Messina, Messina, Italy
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Department of Analytical Chemistry, Faculty of Chemical Technology, University of Pardubice, Pardubice, Czech Republic
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- 12:00 – 13:30 **Lunch break – poster session – poster removal**
- 13:30 – 13:45 **Vratislav Peška**
Mendel Centre for Plant Genomics and Proteomics, Central European Institute of Technology, Masaryk University, Brno, Czech Republic
 BAL31-NGS IN TELOMERE BIOLOGY

- 13:45 – 14:00 **Agnieszka Szmitkowska**
*Central European Institute of Technology, Masaryk University,
Brno, Czech Republic*
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- 14:00 – 14:15 **Sona Valuchova**
*Central European Institute of Technology, Masaryk University,
Brno, Czech Republic*
A NOVEL RAPID METHOD FOR DETECTING PROTEIN-
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- 14:15 – 14:30 **Monika Skrutková Langmajerová**
*Department of Biochemistry, Faculty of Science, Masaryk
University, Brno, Czech Republic; Institute of Organic and
Analytical Chemistry, University of Orleans and the French
National Center for Scientific Research, Orleans, France*
CAPILLARY ELECTROPHORESIS WITH ONLINE
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Peter Troška, Lucia Chropeňová, Marián Masár
- P72 CE-nESI/MS IN ELECTRODE-FREE DESIGN: NARROWING THE SEPARATION CHANNEL TO 5 μm
Anna Tycova, Frantisek Foret
- P73 MICROFLUIDIC DEVICE FOR CELL COUNTING AND CHARACTERIZATION
Tomas Vaclavek, Jana Krenkova, Frantisek Foret
- P74 PREDICTION OF GRADIENT RETENTION DATA FOR HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHIC SEPARATION OF NATIVE AND FLUORESCENTLY LABELED OLIGOSACCHARIDES
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Pavčina Víšková, Lola Bajard-Ešner, Lukáš Trantírek, Silvie Trantírková, Jan Ryněš
- P77 ANALYSIS OF INORGANIC POSPHORUS OXYANIONS BY CAPILLARY ZONE ELECTROPHORESIS
Sebastian Pallmann, Jana Šteflová
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Jana Steflová, Martin Stefl, Sarah Walz, Oliver Trapp

About the invited speakers



Jana Krenkova is a Staff Scientist at the Department of Bioanalytical Instrumentation, Institute of Analytical Chemistry of the Czech Academy of Sciences in Brno. She received her M.Sc. degree in Analysis of Biological Materials (2003) and Ph.D. degree in Analytical Chemistry (2007) at the University of Pardubice, Czech Republic. During years 2007-2010, she was as a postdoctoral fellow at the Lawrence Berkeley National Laboratory, Berkeley, CA, USA working on development of enzyme and nanoparticle-modified monolithic materials for bioanalytical applications. In 2010, she received a Marie Curie Fellowship and joined the group of Frantisek Foret at the Institute of Analytical Chemistry in Brno. Her research interests include monolithic materials, enzymatic reactors, nanoparticle synthesis, and separation/mass spectrometry coupling.



Erdmann Rapp studied chemistry at the universities of Konstanz and Tübingen in Germany. During his PhD, he was stipendiary of DFG graduate school for analytical chemistry at the Eberhard Karls University of Tübingen and did fundamental research and method development on miniaturized separation techniques coupled to MS and NMR. He was invited research fellow at the NMR Centre of Wageningen University (The Netherlands), studying fluid dynamics in miniaturized separation systems via NMR-imaging.

In 2001 he got research associate and head of the Laboratory for Miniaturized Separation Techniques at the Institute of Process Engineering of the Otto von Guericke University in Magdeburg (Germany). He continued his fundamental research on fluid dynamics in electrokinetically and hydrodynamically driven open tubular and packed capillaries and its impact on the analysis of biomolecules.

Since 2003 till present, he is head of Bio/Process Analytics at Max Planck Institute for Dynamics of Complex Technical Systems in Magdeburg (Germany). He and his team are working on the development and implementation of innovative cutting edge bio/process analytical tools for a deeper understanding of bio(techno)logical processes - in particular, on high-throughput tools for proteomics, glycoproteomics and glycomics to be able to handle large sample numbers arising along bioprocess development and biomarker discovery. With the invention of “glyXbox”, a high-performance glycoanalysis system, he got key founder of glyXera GmbH, providing glycoanalytical services to academia, clinics and industry.



Guillaume Erny is a principal researcher in the Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering of the University of Porto in Portugal. He received his PhD in Analytical Chemistry in 2003 from the University of York (UK). He was awarded the Ronald Belcher Award in 2002 (Royal Society of Chemistry, UK) for his work on the HVL function applied to capillary electrophoresis. He then held a postdoctoral position in the Laboratory of Biomolecular Science at UMIST, Manchester, UK; a postdoctoral position at the CSIC, Madrid, Spain; and a postdoctoral position at the University of Aveiro, Portugal. He developed a keen interest in chemometrics approaches applied to the analysis of hyphenated MS dataset and self-taught Matlab in order to test and develop the analysis algorithms. He lives in sunny and windy Aveiro, Portugal, with his wife and two daughters who keep him busy.



Andrew Ewing received a PhD from Indiana University. After 25 years at Penn State University, he is now Professor at Chalmers University and University of Gothenburg, Sweden. His group has pioneered small-volume chemical measurements at single cells, electrochemical detection for capillary electrophoresis, novel approaches for electrochemical imaging of single cells, and new electrochemical strategies to separate individual nanometer vesicles from cells and quantify their contents. His 300 publications have been cited over 16000 times with an H-index of 69. He has recently received the Charles N Reilley Award from the Society for Electroanalytical Chemistry (2013), the ACS Analytical Division Award in Electrochemistry (2013), the Norblad-Ekstrand Medal of the Swedish Chemical Society (2014), and the Pittsburgh Conference Award in Analytical Chemistry (2015). He is an Honorary Professor at both Nanjing University of Science and Technology and Beijing University of Science and Technology. He is a member of the Royal Swedish Academy of Sciences (2012) and the Gothenburg Academy of Arts and Sciences (2013).



András Guttman, MTA-PE Lendulet professor of Translational Glycomics, is the head of the Horváth Csaba Laboratory of Bioseparation Sciences and also leads the application efforts at Sciex. His work is focused on capillary electrophoresis and CESI-MS based glycomics and glycoproteomics analysis of biomedical and biopharmaceutical interests. Dr. Guttman has more than 290 scientific publications, wrote 35 book chapters, edited 4 textbooks and holds 23 patents. He is a member of the Hungarian Academy of Sciences, on the board of several international organizations, serves as editorial board

member for a dozen scientific journals and has been recognized by numerous awards including the Analytical Chemistry Award of the Hungarian Chemical Society in 2000, named as Fulbright Scholar in 2012, received the CASSS CE Pharm Award in 2013, the Arany Janos medal of the Hungarian Academy of Sciences, the Pro Scientia award of the University of Pannonia and the Dennis Gabor Award of the Novofer Foundation in 2014.



During his PhD study, **Steven Ray Wilson** worked with the development of on-line 2D LC systems and the hyphenation of LC and NMR. After his PhD, he became involved in drug discovery efforts through the Cancer Stem Cell Innovation Center. Today, his focus is on applying miniaturized LC-MS systems in cancer diagnostics, working as an Associate Professor at the University of Oslo, collaborating with a number of medical environments.



Radim Chmelík

Education

1997, Ph.D., Faculty of Mechanical Engineering, BUT, Physical and Material Engineering

2002, Associate Professor, Faculty of Mechanical Engineering BUT, Applied Physics

2012, Professor, Faculty of Mechanical Engineering BUT, Applied Physics

Scientific activities

Coherence-controlled and correlation holographic microscopy, quantitative phase imaging, microscopy of three-dimensional objects, microscopy in turbid media, applications of holographic microscopy in living-cell biology, experimental biophotonics

Industry cooperation

TESCAN ORSAY HOLDING, a. s., AREKO, s.r.o., Meopta - optika, s.r.o.

Prizing by scientific community

2013, Werner von Siemens Excellence Award 2013, in category: The most important result of development/innovation

2013, 1st place in Cooperation of the Year competition (AFI, AmCham, TACR)



Kareem Elsayad obtained his Masters Degree and PhD in the UK and the USA respectively in the field of condensed matter physics, before moving to Vienna to work on developing optical microscopy techniques for life science research applications as a postdoctoral researcher. Since the end of 2013 he heads the Advanced Microscopy division of the Vienna Biocenter Core Facilities (VBCF) in Vienna, where he oversees a team of optical physicists and biophysicsts/biologists developing novel and cutting edge optical microscopy solutions for and in collaboration with

life-science researchers primarily at the Vienna Biocenter.

Among other things he is inventor of a technique called Spectrally coded Optical Nanosectioning (SpecON) which utilizes the optical interaction of radiation from fluorophores with a nano-structured surface for axial superresolution imaging with a resolution <10nm. He has also developed and demonstrated the correlative microspectroscopy technique - Fluorescence Brillouin imaging (FBi), which can be used to in parallel map the biochemical constituents and mechanical properties within live cells and tissue.

As head of the Advanced microscopy facility his interests broadly cover all things optical microscopy related in particular microspectroscopy, time-resolved fluorescence techniques, light sheet microscopy and superresolution fluorescence microscopy. He is personally interested and engaged in the field of light-matter interactions and the mechanical and physical properties of biological matter in general, as well as designing & building novel optical microscopy setups that make use of the more unusual properties of light and its interaction with matter.



Stavros Stavrakis is currently a Senior Scientist in the deMello group in the Department of Chemistry and Applied Biosciences at ETH Zurich. He received his B.Sc. in Chemistry and Ph.D in Biophysical Chemistry from the University of Crete (Greece) in 2005. His research was focused on the application of time-resolved vibrational spectroscopies such as FTIR and Raman, applied to enzymatic systems (cytochrome c oxidase) involved in catalytic reactions accompanied with proton/charge

transfer reactions. In 2007 Dr. Stavrakis was awarded an Individual Outgoing Marie Curie Fellowship. As a Marie Curie fellow, he spent three years with Prof. Stephen Quake at Stanford University specializing in single molecule biophysics. The focus of his research was to develop new technologies to improve the throughput of current single molecule DNA sequencing platforms. This covered four broad areas: strategic surface deposition techniques, measuring DNA polymerase kinetics using single molecule FRET, single molecule colloidal lensing and novel surface chemistries for enhanced single molecule signals. The ability to achieve single molecule sensitivity with long working distance objectives enabled high temperature single molecule spectroscopy and the ability to measure the kinetics of a thermophilic DNA polymerase for the first time. As part of his fellowship he also worked as a postdoctoral fellow in Dr. David McGloin's lab at the University of Dundee, developing methods for optical trapping and rotation of single cells in microfluidic chips. His current research interests are focused on applications of single molecule fluorescence detection, and optofluidics in biology. Currently he has a team of postdocs and students developing new microfluidic/optofluidic platforms for single molecule enzymology, high-throughput imaging flow cytometry, fast enzyme kinetics, fluorescence lifetime combined with droplet microfluidics and high-throughput microfluidic single-cell screening platforms.



Peter Ertl holds an engineering degree in Biotechnology (BOKU, Austria), a PhD in Chemistry (Univ. Waterloo, Canada) and received his postdoctoral training as a biophysicist at University of California at Berkeley (US). Additionally, in 2003 Dr. Ertl co-founded a biotech start-up company where he served a number of years as Director of Product Development in Kitchener-Waterloo (CAD) developing benchtop-sized microbial analyzers. In 2005 Dr. Ertl moved to Austria where he worked as Senior Scientist in the Biosensor Technology unit at the AIT Austrian Institute of Technology. In 2016 he was appointed Professor for Lab-on-a-Chip Systems for Bioscience Technologies at Vienna University of Technology, where his research focuses on the development of advanced *in vivo* and *in vitro* diagnostic microsystems for biomedical research.



Blanca H. Lapizco-Encinas is an associate professor in the Department Biomedical Engineering at the Rochester Institute of Technology. Her current research efforts are focused on the development of microscale electrokinetic techniques for the manipulation of bioparticles, from macromolecules to cells. Her main research objective is to develop electrokinetic-based microdevices that would answer the needs of many different applications, such as: cell assessment for clinical/biomedical applications and

microorganism manipulation and detection for food safety and environmental monitoring. Her research work has been funded by the NSF and other funding agencies in the US and Mexico. Her research efforts have received awards from the Mexican Academy of Sciences and the L'OREAL for Women in Science program. The research findings obtained by her group have been presented in numerous international conferences. She serves as reviewer for various international Journals and has served as organizer and session chair for several conferences. She is a Deputy Editor for the Journal ELECTROPHORESIS and has served as Vice-President of the American Electrophoresis Society.



Zdeněk Hurák is a researcher and lecturer at the Department of Control Engineering, Faculty of Electrical Engineering, Czech Technical University in Prague. Web page of his research group is <http://aa4cc.dce.fel.cvut.cz>. He got his master's degree in aerospace electrical engineering (summa cum laude) at Military Academy, Brno, Czech Republic, in 1997. He was awarded Boeing Fellowship in 1999, which supported his 3-month research stay at Iowa State University, Ames, USA. In 2000 he started his Ph.D.

study in control systems and robotics under supervision of Michael Šebek at Czech Technical University in Prague, Czech Republic, and defended it in 2004 with the thesis on 11 optimal control. He was a visiting researcher at TU Eindhoven, The Netherlands, May through December 2008. He was awarded Fulbright's scholarship for a visiting position at University of California, Santa Barbara, February through August 2014. These days he gives graduate lectures on optimal and robust control and undergraduate lectures on modeling and simulation of dynamic systems. He supervises graduate students, and manages research projects with academic and industrial partners. His research interests include algorithms for optimal, robust and distributed control, especially within the polynomial (algebraic) framework, and applications of advanced control schemes to electromechanical systems such as inertially stabilized camera platforms, piezoelectric micro-manipulators and non-contact micro-manipulation using dielectrophoresis. He is a Senior Member of IEEE and serves the community as a chair of Control Systems Chapter of Czech-Slovak Section of IEEE, and as a member of the editorial board of *Automa* journal (and *Kybernetika* journal in the past). He reviews for IEEE TAC, IEEE TCST, IFAC Automatica, IFAC Mechatronics, IFAC Control Engineering Practice and Asian Journal of Control. He (almost) regularly attends IEEE CDC and IFAC World Congress, and very often IEEE MSC, ACC, MTNS, IFAC ROCOND and a occasionally a few other conferences and workshops.



Daniel Georgiev received his Ph.D. from the University of Michigan, Ann Arbor, in 2007 in the area of Systems and control theory. His studies were funded by the National Science Foundation Graduate Research Fellowship and the University of New Mexico Presidential Scholarship. In 2007, he accepted a postdoctoral grant from the National Institute of Health for cross-training in biology. He completed his postdoctoral studies at the

Klavins lab of synthetic biology, University of Washington, Seattle, WA. In 2010, Daniel Georgiev joined the Faculty of Applied Sciences, University of West Bohemia, where he founded the Synthetic biology laboratory (www.ccy.zcu.cz) within the NTIS research and the Department of cybernetics. Most recently, he cofounded XENO Cell Innovations, a startup company focused on commercialization of whole cell biosensor technologies.



Mikhail Gorshkov was graduated from Moscow Institute of Physics and Technology with PhD in Chemical Physics in 1987. His early research interests were in precise atomic mass measurements and mass spectrometry instrumentation. Since then he worked at The Ohio State University (Columbus, USA) and Pacific Northwest National Laboratory (Richland, USA) mainly focused on developing mass spectrometers and mass spectrometry based approaches for studying proteins. In 2002 he founded Laboratory of Physical and Chemical Methods for Structure Analysis at the Institute for Energy

Problems of Chemical Physics, Russian Academy of Sciences. Currently, his laboratory's main areas of research are broadly within mass spectrometry instrumentation, liquid chromatography, and bioinformatics with applications in proteomics.



In 1998 **Jana Roithová** graduated in organic chemistry from the Faculty of Science of Charles University in Prague, she then continued with her PhD studies at the Faculty of Chemical Engineering of the Institute of Chemical Technology, Prague. Her PhD thesis was devoted to reaction dynamics and was completed under the supervision of Prof. Zdenek Herman (J. Heyrovsky Institute of Physical Chemistry). After obtaining the PhD title in 2003, she undertook a postdoctoral stay at the Technical University in Berlin in the group of Prof.

Helmut Schwarz. From 2007 she is working at the Faculty of Science of the Charles University in Prague, where she presently holds a position of full professor and she is

also a head of the department of organic chemistry. She is author of more than 140 papers in peer-reviewed scientific journals.

The major research interests of J. R. involve development of new approaches for studying of reaction mechanisms and reactive intermediates by mass spectrometry. Typically several experimental approaches are combined with computational chemistry for obtaining of a detailed picture of chemical reactivity. She uses infrared predissociation spectroscopy for investigation of reactive ions/ionic intermediates in a cryogenic ion trap and various dedicated mass spectrometry experiments for uncovering of details of elementary reaction steps.

Renato Zenobi is Professor of Analytical Chemistry at the Organic Chemistry Laboratory of the Swiss Federal Institute of Technology (ETH) Zurich. Born in Zurich in 1961, he received a M.S. degree from the ETH Zurich in 1986, and a Ph.D. at Stanford University in the USA in 1990. This was followed by two postdoctoral appointments at the University of Pittsburgh (1990 - 1991) and at the University of Michigan (1991). Renato Zenobi returned to Switzerland in 1992 as a Werner Fellow at the EPFL, Lausanne, where he established his own research group. He became assistant professor at the ETH in 1995, was promoted to associate professor in 1997, and to full professor in 2000. He was chairman of the Organic Chemistry Laboratory in 2002-2003 and 2011-2012, served as the president of ETH's university assembly (Hochschulversammlung, HV) from 2006 – 2008, and of the lecturer's conference (Konferenz des Lehrkörpers, KdL) at ETH Zurich 2006 - 2010. Zenobi was a visiting professor at the Barnett Institute (Boston) in 2004/2005, and at the Institut Curie (Paris) in 2010. In 2010 he was appointed Associate Editor of Analytical Chemistry (American Chemical Society). He has chaired the 2014 International Mass Spectrometry Conference in Geneva, Switzerland.



Zenobi's research areas include laser-based analytical chemistry, electrospray and laser-assisted mass spectrometry, ambient mass spectrometry, and near-field optical microscopy and spectroscopy. He has made important contributions to the understanding of the ion formation mechanism in matrix-assisted laser desorption/ionization (MALDI) mass spectrometry, and to ambient ionization methods. He is well known for the development of analytical tools for the nanoscale, in particular TERS (tip-enhanced Raman spectroscopy), a spectroscopic methodology with ≈ 10 nm spatial resolution.

Renato Zenobi has received many awards for his scientific work, including the Thomas Hirschfeld Award (1989), an Andrew Mellon Fellowship (1990), the Ruzicka Prize (1993), the Heinrich Emanuel Merck-Prize (1998), the Redwood Lectureship from the Royal Society of Chemistry (2005), the Michael Widmer Award (2006), a honorary Professorship at East China Institute of Technology (2007), the Schulich Graduate Lectureship (2009), a honorary membership of the Israel Chemical Society

(2009), honorary professorships at the Chinese Academy of Sciences (Changchun), at Hunan University, and at Changchun University of Chinese Medicine (2010), the Mayent-Rothschild Fellowship (Institut Curie, Paris; 2010), the Fresenius Lectureship from the German Chemical Society (2012), the Thomson Medal (International Mass Spectrometry Foundation, 2014), the 2014 RUSNANO prize, and the 2015 Fresenius Award (German Chemical Society/GDCh).

Abstracts of oral presentations – Invited speakers

A NOVEL NANOSPRAY LIQUID JUNCTION INTERFACE FOR VERSATILE CE-MS

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Summary

Coupling of capillary electrophoresis (CE) with mass spectrometry (MS) represents a powerful combination to perform selective, efficient and sensitive analysis of a variety of compounds. Several interfaces to hyphenate CE and MS have been developed and most of them can be categorized into either the sheath-flow or sheathless arrangement.

In this work we present a new polyimide-based liquid junction nanospray interface, combining the advantages of sheath flow and sheathless designs. The liquid junction interface was prepared as a hybrid microfabricated plastic liquid junction sprayer allowing to perform analysis in standard (30-100 µm ID) separation capillaries. The interface incorporates a self-aligning port for coupling of the separation capillary as well as ports for automated flushing of both the liquid junction and the separation capillary. The interface permits performing CE both with and without electroosmotic flow. The use of large bore capillaries allows injection and preconcentration of larger sample volumes, favoring detection sensitivity. Different compositions of the spray liquid and BGE can be used when needed.

The performance of the interface is demonstrated for analysis of a broad range of analytes such as metabolites, peptides, glycans, and intact proteins, including monoclonal antibodies and their fragments. A comparison with a commercially available sheath-flow as well as sheathless (CESI) interface will be presented, discussing analytical parameters such as repeatability, linearity and sensitivity.

Acknowledgement

Financial support from the Grant Agency of the Czech Republic (16-09283Y, P206/12/G014), and the institutional research plan (RVO:68081715) is acknowledged.

XCGE-LIF: A POWERFUL TOOL IN THE GLYCOANALYTICAL TOOLBOX

Erdmann Rapp^{1,2}, René Hennig^{1,2}, Samanta Čajić¹, Thilo Muth^{1,2}, Robert Kottler¹, Udo Reichl^{1,3}

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Summary

Glycomics is a rapidly emerging field that can be viewed as a complement to other „omics“ approaches. Hence, there is a dramatic increase in the demand for analytical tools and specific databases in glycobiology, respectively, glycobotechnology. In order to enhance and improve the comparatively small existing glycoanalytical toolbox [1], fully automated, highly sensitive, reliable, high-throughput and high-resolution analysis methods including automated data evaluation are required.

Our glycoanalysis approach, based on multiplexed capillary gelelectrophoresis with laser induced fluorescence detection (xCGE-LIF) shows high potential for high-performance analysis of glycoconjugates. The development of this high-performance glycoanalysis system (method, software and database) and its application to different fields with respect to sample preparation, separation and automated data analysis is presented [2-6]. The smart applicability of the system is demonstrated for different types of glycosamples (biopharmaceuticals, vaccines, human milk and blood serum) [1-8]. This novel modular high-performance glycoanalysis system allows fully automated, highly sensitive, instrument-, lab- and operator-independent "real" high-throughput glycoanalysis. This is in contrast to the currently prevailing methods, where high-throughput is highly cost and lab-space intensive and ties up a lot of manpower.

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UNLEASHING UNTARGETED HYPHENATED MS ANALYSIS WITH CHEMOMETRICS

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Summary

The use of mass spectrometers (MS) as a detector has been a true game-changer in analytical separation techniques. Hyphenated MS techniques not only allow an easy identification of the main peaks, but, when used as the detector, MS has the unique ability to be simultaneously a near universal detector and extremely selective. Yet, while hyphenated MS techniques are unrivalled for targeted approaches, they do not easily allow a comprehensive analysis of unknown samples. This is mainly due to the large amount of noise that may hide relevant information, as well as the redundancy of signals generated by each compound entering the detector. This results from the ionisation mode: any chemicals entering the chamber will produce multiple ions and therefore will leave multiple footprints in the dataset that could be wrongly assigned to different compounds.

Chemometrics approaches can help in extracting, organising and visualising the information that is contained within a dataset. We recently developed such an approach, called clusters plot that allows a comprehensive analysis when using a separation technique hyphenated with high resolution mass spectrometry (X-HRMS). Briefly, after extracting all series of points (profiles) that are related to the transport and ionisation of a compound, a clustering analysis is used to classify all profiles based on their similarities. The aim is to group similar profiles in a cluster, where each cluster will regroup all the profiles related to one and only one compound.

The clusters plot is obtained after running a series of independent functions programmed using Matlab. All these functions are in open access and can be downloaded from <https://finneeblog.wordpress.com/>. This presentation aims at demonstrating the potential of this approach, as well as discuss its different assumptions and possible limitations. The presentation will be illustrated using deproteinized urine samples that were analysed using capillary electrophoresis hyphenated with time of flight mass spectrometry.

ANALYTICAL MEASUREMENTS WITH CAPILLARY ELECTROPHORESIS, FROM ELECTROCHEMICAL CYTOMETRY TO ALZHEIMER'S PEPTIDE IN CSF

Dimitri Brinet¹, Johan Dunevall², Yasmine Iacone¹, Jörg Hanrieder¹, Andrew Ewing^{1,2}

¹*University of Gothenburg, Gothenburg, Sweden*

²*Chalmers University of Technology, Gothenburg, Sweden*

Summary

Capillary electrophoresis with electrochemical detection (CEEC) has been used to develop methods to measure neurotransmitter storage in single cell vesicles with a method we called electrochemical cytometry and led to a new separationless method for electrochemical impact cytometry of nanometer vesicles. Additionally, we are using CEEC for analysis of the Alzheimer's-related Amyloid Beta (A β) peptide aimed at analysis in cerebral spinal fluid of patients. Here, CE is critically important to separate the different A β species and this has not been done before in cerebral spinal fluid samples.

Measuring the contents of individual nanometer transmitter vesicles is an analytical challenge and important to understand neurotransmission. We developed electrochemical cytometry to separate nanometer vesicles, lyse them on an electrode surface, and amperometrically detect the active contents of each vesicle in a high throughput manner. This led to a new method of electrochemical cytometry where the electrochemical response to single adrenal chromaffin vesicles filled with hormone transmitters as they impact a 33- μ m diameter disk-shaped carbon electrode can be measured. This has been used to compare the amount in vesicles to the amount released and then to argue that the predominant mode of exocytosis is not all-or-none, but rather open and closed and the vesicles are reused.

Alzheimer's disease is a progressive disorder characterized, in part, by deposition of amyloid peptides made primarily of the A β peptide. These A β peptides are characterized by sequential cleavages of the amyloid precursor protein. These peptides can, however, assemble into possibly neurotoxic structures that might be the cause of the disease symptoms (1). A major difficulty in understanding the role of these structures is the challenge associated with isolating and monitoring these metastable species. We will present a label free CEEC method to separate, identify, and measure these species.

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GLYCOHISTOPATHOLOGY: MINING THE FFPE DEPOSITORIES BY CAPILLARY ELECTROPHORESIS

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Pannonia, Veszprem, Hungary*

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Summary

Glycohistopathology is a subset of the emerging field of molecular pathology to utilize the large number of tissue biopsy samples that are collected yearly for histology based diagnostics and archived in formalin-fixed and paraffin-embedded (FFPE) forms. Buffered formalin is the most commonly used fixative, which crosslinks the amino groups of proteins through methylene bridges, providing preservation to maintain the structural integrity of the specimens. Although FFPE samples have been used for molecular pathology studies before, such as for DNA analysis, little information is available on the effect of formalin-fixation on the sugar structures of the glycoproteins in these samples. In this presentation we report on the use of capillary electrophoresis with both laser induced fluorescence (LIF) and mass spectrometry (MS) detection methods to profile the released N-linked glycans from standard glycoproteins and tissue biopsy samples before and after the formalin-fixation and paraffin-embedding processes. The released carbohydrates were fluorophore labeled before analysis with a triple charged APTS tag. The resulting N-glycome patterns were very similar before and after formalin fixation and paraffin embedding. The low sample requirement of CE-LIF and CESI-MS was very useful to analyze miniscule sample amounts from the FFPE slides. To obtain detailed structural information, exoglycosidase based carbohydrate sequencing was also applied using. The sequencing results were verified by CESI-MS. Based on our preliminary results, FFPE samples represent a rich source for glycobiomarker discovery, especially with the associated clinical outcome information. In addition, this approach represents an unparalleled opportunity for retrospective and prospective studies in both biomedical and pharmaceutical interests.

OPEN TUBULAR LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY: A POWERFUL AND VERSATILE TOOL FOR BIOANALYSIS

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Summary

For decades, open tubular liquid chromatography (OTLC) has promised excellent chromatographic performance. However, technical challenges obstructed its development, as well as the advances of other LC formats, making one ask “what do we need OTLC for anyway?”

However, in biomedical research, there is an increasing need for analyzing very limited biosamples, e.g. stem cells and disease-promoting extracellular vesicles such as tumor-derived exosomes. Here, OTLC may find a home, as it has in later years provided unprecedented sensitivity and is very well matched with nanospray-mass spectrometry (nESI-MS).

In our group, which focuses on e.g. stem cell-ness in cancer, OTLC-MS has recently been used for comprehensive and targeted proteomics, using an organic polymer layer on the inner walls of the columns as a stationary phase. However, we find that OTLC columns featuring a silica-based layer are highly versatile, allowing highly efficient separations for bottom-up proteomics as well as intact proteins. In addition, these columns allow for high resolution, attogram level detection of small molecules, such as breast cancer-related lipids. Coupled with on-line enzymatic reactors (also open tubular), OTLC can allow detection of trace analytes in just a few hundred cells.

OTLC systems are also being applied in other research areas, such as an analysis tool in counter-terrorism. In other words, OTLC is a tool that is beginning to show strong potential for practical use in several application areas.

LIVE-CELL QUANTITATIVE PHASE IMAGING BY COHERENCE-CONTROLLED HOLOGRAPHIC MICROSCOPY

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Summary

Quantitative phase imaging (QPI) is one of modern microscopical techniques that has emerged during last decades and substantially improved possibilities and quality of biomedical imaging. Image contrast in QPI originates from phase shifts of light introduced by an observed specimen. For this reason, high-contrast imaging of live cells is achieved non-invasively and without application of possibly phototoxic markers. The phase shifts measured by the QPI in individual image pixels are proportional to the dry-mass density in the corresponding points of observed cells [1]. Thus the distribution of mass in a cell and its changes over time can be observed and measured for hours and days. The advantage of coherence-controlled holographic microscopy (CCHM) equipped with incoherent-light source consists in one-shot imaging (similarly to wide-field) with clean background without any coherence artefacts, which provides a high accuracy of mass measurements. This is why CCHM suits ideally cell growth assessment as well as evaluation of their motility based on protrusion/ retraction measurement. In addition, the coherence-gate effect makes possible QPI of live-cell behavior in turbid or complex 3D milieu. We demonstrate the usage of the CCHM QPI qualities. The appraisal of cellular responses to the course of sub-toxic challenges even in turbid medium is substantially improved. The kind of cell death and modifications of cell behavior by pathological processes can be revealed.

Acknowledgement

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UNRAVELLING AND UNDERSTANDING THE MECHANICAL PROPERTIES OF PLANTS USING BRILLOUIN LIGHT SCATTERING MICROSPECTROSCOPY

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Summary

Brillouin Light Scattering (BLS) spectroscopy is an all-optical label-free technique which allows for the determination of the viscoelastic properties of a sample. BLS is generally a very weak process, based on the interaction of light with thermal density fluctuations, and thus challenging to implement for lifescience/biomedical applications. Recent advances in spectrometer and camera designs have however made it possible to perform BLS measurements on live cells, opening the door to a new means of studying the mechanical properties of biological systems. Here I will discuss the use of BLS Microspectroscopy and correlative Fluorescence – BLS Microspectroscopy to map the viscoelastic properties of cells and tissue in 3 dimensions, focusing on its use to understand the mechanical properties of plant cells. 3 dimensional mapping of the mechanical properties of plant cells is particularly interesting given the delicate balance between extracellular matrix (cell wall) mechanical properties and turgor pressure involved in defining cell shape, assuring “correct” development, for maintaining the structural integrity of the organism as a whole, and ultimately determining their survival subject to all types of environmental perturbations.

Firstly I will give an introduction to BLS including experimental setups and the physical principles it is based on. I will then discuss some details of the quantities that are and can be extracted from a BLS measurement and how and to what extent they may be compared to or compliment results obtained from alternative measurements of the mechanical properties of and within cells - such as those obtained using microrheology and perturbation-deformation techniques such as Atomic Force Microscopy (AFM). I then will present a series of studies on different live plant cells and tissue we have performed focusing on the physical and biological significance of the obtained results. Finally I will summarize the strengths of the technique, its limitations and some of the current challenges, along with an outlook of what we are working on, and some planned and potential future applications in biophysics research as well as medical diagnostics.

DROPLET-BASED MICROFLUIDICS: HIGH-THROUGHPUT EXPERIMENTATION ONE DROP AT A TIME

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Summary

The past 25 years have seen considerable progress in the development of microfabricated systems for use in the chemical and biological sciences. Interest in microfluidic technology has driven by concomitant advances in the areas of genomics, proteomics, drug discovery, high-throughput screening and diagnostics, with a clearly defined need to perform rapid measurements on small sample volumes. At a basic level, microfluidic activities have been stimulated by the fact that physical processes can be more easily controlled when instrumental dimensions are reduced to the micron scale [1]. The relevance of such technology is significant and characterized by a range of features that accompany system miniaturization. Such features include the ability to process small volumes of fluid, enhanced analytical performance, reduced instrumental footprints, low unit costs, facile integration of functional components within monolithic substrates and the capacity to exploit atypical fluid behaviour to control chemical and biological entities in both time and space.

My lecture will discuss recent studies that are focused on exploiting the spontaneous formation of droplets in microfluidic systems to perform a variety of analytical processes.

Droplet-based microfluidic systems allow the generation and manipulation of discrete droplets contained within an immiscible continuous phase [2]. They leverage immiscibility to create discrete volumes that reside and move within a continuous flow. Significantly, such segmented-flows allow for the production of monodisperse droplets at rates in excess of tens of KHz and independent control of each droplet in terms of size, position and chemical makeup. Moreover, the use of droplets in complex chemical and biological processing relies on the ability to perform a range of integrated, unit operations in high-throughput. Such operations include droplet generation, droplet merging/fusion, droplet sorting, droplet splitting, droplet dilution, droplet storage and droplet sampling [3, 4]. I will provide examples of how droplet-based microfluidic systems can be used to perform a range of experiments including nanomaterial synthesis [5], cell-based assays [6] and DNA amplification [7]. In addition, I will describe recent studies focused on the development of novel imaging flow cytometry platform that leverages the integration of inertial microfluidics with stroboscopic illumination [8] to allow for high-resolution imaging of cells at throughputs approaching 10^5 cells/second.

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ORGAN-ON-A-CHIP TECHNOLOGY: MONITORING DYNAMIC CELL RESPONSES UNDER CONTROLLED PHYSIOLOGICAL CONDITIONS

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Summary

Microfluidics is vital for cell analysis because it is the only technology capable of simulating the physiological environment of cells and cell assemblies to investigate cellular transport mechanisms and cell proliferation events in the presence of test reagents, temperature or shear force gradients. In light of the benefits of microfluidics, my research group at TUW is developing lab-on-a-chip systems containing integrated fluid handling, degassing and biosensing systems to non-invasively monitor dynamic cell population responses. We have successfully integrated different electro-analytical, magnetic and optical detection methods in microfluidic devices (a) to assess the behaviour of structured 3-dimensional *Candida* biofilms, (b) to monitor dynamic stress responses of mammalian cell populations and (c) to detect cell-to-cell and cell-to-matrix interactions. In course of the presentation various components including microvalves, micropumps, degassers and sensing systems for lab-on-a-chip will be presented as well as their potential for automated, miniaturized and integrated multilevel cell analysis will be discussed.

PARTICLE CAPTURE AND ENRICHMENT EMPLOYING NON-UNIFORM ELECTRIC FIELDS AND INSULATING STRUCTURES

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Summary

Microfluidics is a dynamic and rapidly growing field with potential applications in numerous areas, from food and water safety, to environmental monitoring and clinical analysis. Working on the microscale offers attractive advantages such as shorter processing times, low sample and reagent consumption and the possibility of portable systems with a high level of integration. Manipulation and analysis of biological particles is one of the fields that has been most benefited with the marriage of microfluidics and analytical sciences. Many of these applications require fast response methods able to handle/analyze high value biological products, in a gentle manner, without leading to cell damage or bioproduct denaturation.

Important research efforts are being devoted to the development of analytical techniques that can be used with microfluidic devices. Electrokinetics, electric field driven techniques, such as electrophoresis, electroosmosis and dielectrophoresis, are widely used in microfluidic devices, offering new ways to assess, manipulate and analyze bioparticles. Dielectrophoresis (DEP) is an electrokinetic transport mechanism driven by polarization effects when a dielectric particle is exposed to a spatially non-uniform electric field. DEP has great flexibility, since it can be used with charged and neutral particles employing AC or DC electric potentials. DEP offers more versatility than electrophoresis since it has the capability for significant particle enrichment, up to three orders of magnitude. DEP also offers means for effective continuous particle sorting.

This work is focused on insulator based DEP (iDEP), a dielectrophoretic mode that uses insulating structures between two external electrodes to create electric field gradients and generate dielectrophoretic forces on particles. This study includes experimental and mathematical modeling work. Particle mixtures, including biological cells, were separated and analyzed employing novel variations of iDEP. The results demonstrate that iDEP has great potential as a bioanalytical technique.

SINGLE-PARTICLE HIGH-PRECISION MICROMANIPULATION USING DIELECTROPHORESIS

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Summary

The talk will start by giving an overview of basic principles and state of the art in noncontact manipulation with one or several (micro)particles by shaping force fields of various physical origin. Typically, physical phenomena related to electricity and magnetism are exploited, in which case electric voltages applied to a regular array of (micro)electrodes or electric currents flowing through a regular array of coils are controlled. A promising physical phenomena for bioanalytical application is dielectrophoresis. The dielectrophoretic force derives from an electric field, thus the particle need not respond to a magnetic field. Neither does the particle have to be electrically charged. Although applicability of dielectrophoresis to sub-micron size particles has been demonstrated, dielectrophoresis seems particularly suitable for particles of sizes around a micron and larger, hence a candidate for a biological cell manipulation technique. Indeed, use of dielectrophoresis for manipulation of cells has been documented many times in the literature, dating back to the pioneering work of Herbert Pohl in 1950s. The currently prevailing modes of use of dielectrophoresis are separation and characterization, in which cases a bulk of microparticles is manipulated at once. In this talk, however, we will focus on the use of dielectrophoresis for a high-precision manipulation of a single particle (single cell). After introducing the state of the art, we present our own results in this domain of single-particle manipulation using dielectrophoresis. Namely, we will show how a clever yet simple microelectrode layout in combination with a real-time optimization procedure can achieve high-accuracy steering of a microparticle all over a flat surface, with the range of motion stretching up to a few millimeters. We will discuss the physical principles, fabrication issues and the control-algorithmic tricks upon which our solution rests. The functionality of the experimental platform developed by our team has been demonstrated in various scientific competitions: 4th and 5th place in IEEE RAS Mobile Microrobotics Challenge in 2012 and 2013, respectively, 1st place in Matlab and Simulink Student Design Challenge in 2013 (this one was based on magnetophoresis, though, but it is related). Numerous videos will accompany the talk. Our ultimate motivation for the presented research is to contribute to a development of a low-cost, fully automated and possibly disposable bioanalytical platform. We hope this talk might help recognize the potential of bridging the scientific domains of biology and chemistry and engineering domains of controls, automation and robotics.

MANAGING CELL HETEROGENEITY IN MICROFLUIDIC DESIGN

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Summary

Design of microfluidic devices and their integration in lab-on-chip concepts must consider phenotypic heterogeneity. Herein we take a look at heterogeneity in dielectrophoretic (DEP) response within an isogenic population. DEP is a highly sensitive method for non-invasive cell manipulation and monitoring. Cells exposed to non-uniform electric fields are acted on by DEP forces that are frequency-dependent and vary according to the cell internal state. Knowledge of a priori DEP heterogeneity attributed to sample preparation, spontaneous mutation, and non-uniform sample composition can be used to design cell screening applications (e.g., for blood typing or rare cell quantification). Knowledge of induced DEP heterogeneity attributed to cell-cell interaction, cell induction, and microfluidic environmental effects can be used to design integrated devices that characterize dynamic behaviors (e.g., multidrug resistance) that are otherwise difficult to measure. We've developed a method, called DEP cytometry, for quantifying both a priori and induced cell heterogeneity. Similar to flow cytometry that yields fluorescence histograms at given wavelengths, DEP cytometry yields DEP force histograms at given electrical field frequencies. Preliminary experimental data collected for wild type and mutant *Saccharomyces cerevisiae* populations illustrate the high throughput and sensitivity of the method. Differences between yeast strains can be detected with throughput of thousands of cells per minute in a PDMS based prototype device. The experimental method is complemented by developed in silico tools, whereby spatiotemporal distribution of cell states can be predicted for a rich set of microfluidic topologies. Jointly, the experimental and simulation tools enable design of microfluidic devices with inherent consideration for a priori and induced cell heterogeneity.

PROTEOGENOMICS AND SOME OF THE PITFALLS OF THE PROTEOMIC SIDE OF THIS COIN

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Summary

Shotgun proteomics results in identification of tryptic peptides by searching tandem mass spectra (MS/MS) against genome-centered protein database. In the search, a

reference database is typically employed that leaves coding genome variants behind. Thus, the proteins with single amino acid substitution may be left behind the search. In general, a use of one or more custom DNA and/or mRNA sequence databases for LC-MS/MS data search is becoming a growing trend in identifying the encoded variants of amino acid sequence originated from single amino acid polymorphism or alternative splicing. This area of research including the studies on genome re-annotations using proteomic data, is often referred to as proteogenomics. One of its objectives is extracting the biologically relevant information often hidden in the shotgun mass spectrometry data using the customized protein database containing proteins with residue substitutions due to gene mutations. Cancer proteogenomics is an important example because of a large amount of biologically relevant non-synonymous somatic mutations across the tumor genomes. These mutations can be revealed at the proteome level using the bottom-up LC-MS/MS analysis through identification of the so-called variant proteolytic peptides. These are the peptides which correspond to the part of the protein where the residue substitution event occurred due to the gene mutation. However, this approach aiming at identification of variant peptides using customized database searches of shotgun proteomic data is facing a number of challenges including a dilemma of selecting the most efficient database search strategy, sample preparation artifacts, false discovery rate estimation, and mutant protein quantification, among the others. In this talk we will highlight some of these issues using the results of our recent studies as well as the literature data.

The results considered in this talk were obtained with financial support from Russian Science Foundation, grant #14-14-00971 and in collaboration with Prof. Sergei Moshkovskii's lab from The Institute of Biomedical Chemistry, Russian Academy of Sciences.

HELIUM TAGGING INFRARED PHOTODISSOCIATION SPECTROSCOPY

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Summary

Infrared photodissociation spectroscopy became one of the standard tools in determination of the structure of the ions in the gas phase. Next to the often applied infrared multiphoton dissociation spectroscopy, many approaches based on the messenger technique have been developed. We are using helium tagging which permits investigation of highly reactive ions [1].

Helium tagging infrared photodissociation spectroscopy is performed with a tandem mass spectrometer containing a wire quadrupole trap cooled to 3 K. Mass-selected ions are trapped with helium buffer gas and form helium complexes.

Spectroscopy can be performed with tunable light sources in IR or VIS spectral range [2, 3].

I will show application of our approach for the investigation of hyper-valent metal-oxo complexes. We can routinely detect metal-oxo stretching vibrations (among others, I will show the first detection of copper(III)-oxo stretching mode). Recently, we have also demonstrated that we can spectroscopically distinguish triplet and quintet states of the iron(IV)-oxo complexes [4].

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ON-LINE ANALYSIS OF EXHALED BREATH VIA SECONDARY ELECTROSPRAY IONIZATION MASS SPECTROMETRY

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Summary

Exhaled breath contains relevant information on a person's health status. Our vision is to use real-time and completely non-invasive chemical analysis of exhaled breath for applications such as medical diagnosis, monitoring progress and treatment of diseases, drug compliance, pharmacokinetics, and others. The methodology we use to analyze breath in real time is based on secondary electrospray ionization coupled to high-resolution mass spectrometry (SESI-HRMS). It affords ppb-ppt limits of detection, and analysis of compounds with molecular weights up to 1000 Da.

A number of interesting questions can now be addressed via on-line mass spectrometric analysis of exhaled breath: is there a core pattern for individual phenotypes visible in mass spectrometric “breathprints”? Can diurnal changes be monitored via exhaled breath? Can diseases be diagnosed via exhaled breath, and if yes, which ones? Can proper drug use (or drug abuse) be detected via analysis of the chemical composition of exhaled breath? The presentation will focus on several examples in medical diagnosis, including the detection of novel biomarkers for diseases such as obstructive sleep apnea (OSA) and chronic obstructive pulmonary disease (COPD). Monitoring of drug compliance and pharmacokinetics via real-time SESI-MS will also be shown.

Abstracts of oral presentations – CECE Junior 2016

DETERMINATION OF COUNTERION IN CANCER TREATMENT DRUG BY MICROCHIP ELECTROPHORESIS

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Summary

This work is focused on studying the basic methodological aspects of quantitative analysis in isotachopheresis (ITP) on a microchip with integrated conductivity detection in order to achieve high-precise results. From the application point of view, determination of acetate, as an anionic macroconstituent in pharmaceutical preparation busserelin acetate, was studied. The robustness of the ITP quantitative analysis was studied in terms of impact of fluctuations in various working and separation conditions, e.g., fluctuations in the driving current, concentration of the leading ions, pH of the leading electrolyte (LE) and impurities in the terminating electrolyte (TE), on the precision of determination. Additionally, quantitation of acetate was carried out using external calibration and internal standard methods. The recovery values ranging 98-101% indicate accurate determination of acetate in busserelin acetate.

1 Introduction

A salt form of the drug is often used in the drug development. Various inorganic and organic ions can be used as pharmaceutical counterions mainly to improve physicochemical properties of the drug. Chloride, sulfate and acetate are most frequently used counterions for basic drugs [1].

Capillary electrophoresis (CE) is a powerful analytical technique used in the drug development and quality control due to its high separation efficiency and resolution, short analysis time, small sample consumption, relatively simple instrumentation and possibility for coupling to high-sensitive detection techniques [2]. CE methods were already applied to the determination of active drug content, counterions and impurities in pharmaceuticals [3, 4]. In addition to use of conventional CE methods, interest in applying microchip electrophoresis (MCE) to the analysis of biological and pharmaceutical samples is evident [5].

In this work, a methodology to obtain reliable quantitative data from ITP analysis of pharmaceutical preparation performed on the microchip with conductivity detection is described.

2 Experimental

Two identical laboratory-designed MCE equipments were used in this study. Separations were carried out on three identical poly (methylmethacrylate) microchips with coupled channels and integrated conductivity sensors (IonChip™ 3.0, Merck, Darmstadt, Germany). The MCE equipments 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 electrolyte and sample solutions to the microchip and to hydrodynamically close separation system after solutions were loaded. 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 instrument to a PC. This unit also included the measuring electronics of the contact conductivity detectors.

Monitoring of the analysis as well as automatic preparation of the run and collecting the data from conductivity detectors and their evaluation were done using MicroCE Win software, version 2.4 (Merck).

For the preparation of the electrolyte and model sample solutions chemicals of p.a. purity were used. Stock solutions of acetate, succinate, sulfate, and adipate were prepared at a 1 000 mg L⁻¹ concentration. Model and real samples were prepared in a 10% (v/v) TE. Buserelin acetate sample (Merck) was prepared at 18.554 g L⁻¹ and was approximately 10-20 times diluted prior to analysis.

3 Results and Discussion

ITP separations were carried out in a chloride – caproate electrolyte system at a pH of approximately 6.1. Hydrodynamic and electroosmotic flows in the separation system on the microchip were suppressed. Two different quantitative methods were employed for investigating the methodological aspects of the quantitative analysis of acetate in microchip ITP: external calibration and internal standard methods, with succinate as an internal standard.

RSD of the acetate zone lengths ranged from 3 to 4% independently on the microchip or MCE equipment when external calibration was used for quantitation. However, by employing internal standard RSD significantly decreased (from 0.1 to 0.7%). These results indicate high precision of the developed method.

The long-term validity of the ITP quantitative parameters for determination of acetate on two microchips and two MCE equipments was verified. Data obtained show very good possibility for chip-to-chip and/or equipment-to-equipment transfer of the analytical method.

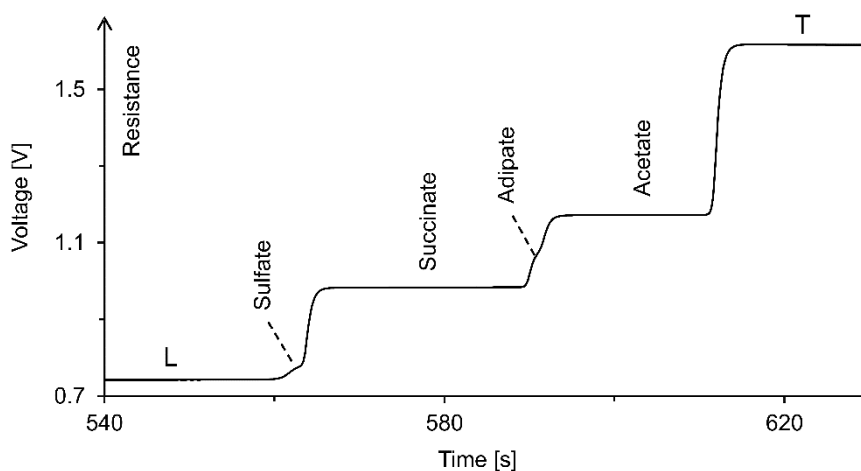


Fig. 1. ITP separation of acetate, succinate and anionic microconstituents present in the TE buffer (1 mg L^{-1} sulfate and 3 mg L^{-1} adipate). Loaded sample contained 60 mg L^{-1} acetate and 100 mg L^{-1} succinate. L leading ion; T terminating ion.

Fluctuations in various working and separation conditions were used for the robustness study. Impact of following fluctuations was studied: (i) driving current, (ii) concentration of the leading ion without changing the pH of the LE buffer, (iii) pH of the LE buffer, and (iv) impurities present in the TE buffer. These parameters were chosen to simulate the actual experimental conditions and errors that can occur in the ITP quantitative analysis on the microchip. Robustness of the ITP quantitation was evaluated from the change of the parameters of the calibration curves for acetate using both external calibration and internal standard methods. The fluctuations in the driving current most significantly influenced the slope values, when the external calibration method was used for calculation (RSD = 8.7%). By comparing the RSD of the slopes of calibration curves for acetate, including all studied fluctuations of the experimental conditions for both methods of quantitation, it is evident that correction on the internal standard improved RSD values of the slope approximately six times.

The study on impurities present in the TE was performed independently of other experimental fluctuations tested because this type of fluctuations could not affect equally analyte and internal standard. The internal standard method reduced the fluctuation in the slope of acetate only two times (approximately two times lower RSD).

Three samples with the addition of different concentrations of acetate were analyzed to evaluate recovery data. Recovery values between 98 and 101% indicate that a very good accuracy of the ITP determination of acetate in buserelin acetate on the present microchip can be expected.

4 Conclusions

This study was showed that ITP on the microchip is a method suitable for the high-precise determination of acetate, main constituent and counterion in the pharmaceutical preparation buserelin acetate. Developed ITP method performed on the microchip with

conductivity detection is suitable for reliable determination of main constituents in relatively simplified pharmaceutical preparations.

Acknowledgement

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ON-CHIP HPLC WITH INTEGRATED DROPLET MICROFLUIDICS FOR FURTHER DOWNSTREAM PROCESSES

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Summary

We present the first integration of droplet-based microfluidics with chip-based high pressure liquid chromatography. The chromatographically separated bands are subdivided into numerous fractions due to the compartmentalization. Fluorescence spectroscopy was employed as detection method. Separation and encapsulation of the analytes are followed by the addition of a fluorescence quenching agent and analysis of the altered fluorescence intensity.

1 Introduction

Chromatographic resolution is not easy to conserve in micro-scale separations due to diffusion and band broadening effects. Collecting the column eluate for further processing, storage or additional analysis is a great challenge because of the occurring remixing. When combining droplet microfluidics with on-chip separation, the loss of individual band resolution can be prevented.

By utilising the herein presented approach it is possible to seamlessly integrate droplet microfluidics with chromatographic separation on a single device with additional downstream analysis of the separated substances.

One of first pioneering works which was published, described the compartmentalization of electrophoretically separated components by means of droplet microfluidics [1]. In the field of chip chromatography one first promising approach using a thermoset polyester (TPE) chip has been published [2]. However, the rather low

pressure resistance of the polymer material limited the achievable chromatographic performance.

Segmented flow systems offer advantages over continuous micro flow systems such as effective convective mixing, isolation of droplet content and avoidance of diffusive zone broadening, which enables long-term storage [3].

A wide range of manipulation techniques exists for droplets such as controlled coalescence, splitting, mixing or extraction of the droplet content [4].

2 Experimental

The developed glass chips were fabricated in-house. Common photolithography followed by a wet-etching procedure and high temperature bonding was employed. High-pressure connection was provided by homemade steel connection clamps. In order to immobilize the column material two porous polymer frits were introduced into the channel via laser-assisted photopolymerization to retain the particles in between. Integration of the column was performed via a slurry packing method based on our previous work [5]. The channels of the low pressure part of the chip, where droplet generation, transport and reagent addition takes place, were modified by treatment with a perfluorosilane.

3 Results and Discussion

Polycyclic aromatic hydrocarbons were separated under reversed-phased conditions and compartmentalized into a sequence of droplets using a T-junction design (MeCN/water, 70%/30%, v:v as mobile phase).

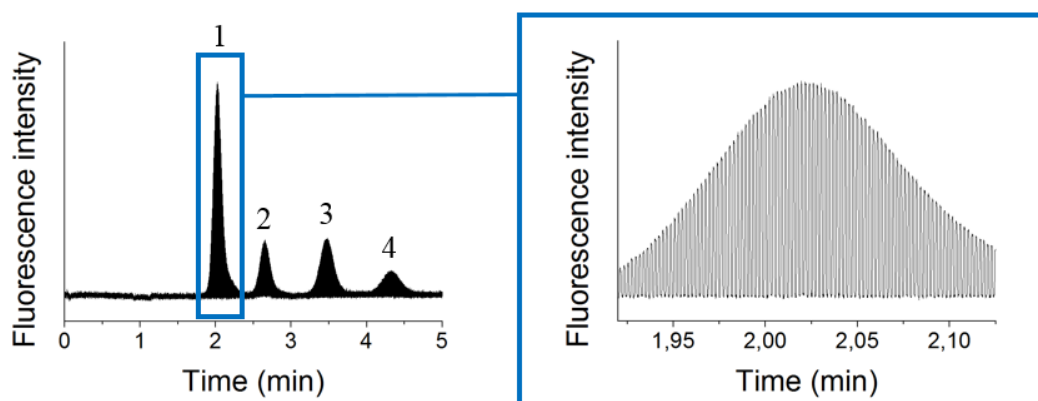


Fig. 1. (left) Chromatogram after a separation of (1) 7-Amino-4-methylcoumarin, (2) Anthracen-9-carbaldehyd, (3) Fluoranthene, (4) Benz(a)anthracene. ProntoSilC18 column 3 μm , column length: 5 cm; mobile phase: 70 % acetonitrile/30% water; eluent flow: 2.5 $\mu\text{L}/\text{min}$, oil flow: 2 $\mu\text{L}/\text{min}$. (right) Section of the first peak illustrating the segmentation into droplets.

Segmentation of the eluent flow is performed by introducing the immiscible oil phase (perfluorodecaline) immediately following chromatographic separation. By coupling the two functionalities, separation and droplet generation, the analytes are

encapsulated into numerous discrete volumes, which are isolated by the surrounding oil phase. The compartmentalization preserves the analytical resolution since the continuous phase restricts dispersion between the droplets. Moreover, the droplets are ideal vessels to perform additional analysis or reactions as each droplet can be viewed as an individual micro reactor.

To demonstrate the suitability for further downstream analysis a quenching agent was added to each droplet via an integrated dosing unit. The resulting decrease in signal intensity was monitored by fluorescence spectroscopy again.

4 Conclusions

In this work we have shown the compartmentalization of a micro scale LC separation followed by downstream addition of a quenching reagent and analysis of its impact on the respective droplet content.

The variability of the chip designs allows for integration of even more functionalities such as an upstream reaction, droplet sorting or the hyphenation with other analytical techniques like mass spectrometry.

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THE MALDI IONIZATION AS A TOOLS OF MODERN BIOANALYTICS

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Summary

The aim of this work was to use matrix-assisted laser-desorption/ionization time-of-flight and tandem time-of-flight mass spectrometry (MALDI-TOF MS) as an analytical tool in study of microbial cells, low-molecular compounds and as well proteins.

1 Introduction

Rapid detection and identification of microorganisms is a challenging and important aspect in many areas of our life, beginning with medicine, ending with industry. Unfortunately, classical methods of microorganisms' identification are based on time-consuming and labor-intensive approaches [1]. Screening techniques require rapid and cheap grouping of bacterial isolates, protein or drugs however modern bioanalytics demands comprehensive bacterial and proteomics studies on molecular level. To meet these requirements matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been proposed as a technique of choice for quick classification of bacterial cells, precise characterization of microorganisms and as well tools for identification low-molecular compounds and proteins [2, 3].

2 Experimental

All mass spectrometry analysis were acquired on a Bruker Ultraflex mass spectrometer (Bruker Daltonics, Bremen, Germany). MALDI-TOF/TOF MS was equipped with a N₂ laser smartbeam II (337 nm, frequency 2000 Hz). The mass spectrometric analysis was performed in three different modes: reflection, linear and post-source-decay (PSD). Reflection spectra were calibrated with the PAC II Peptide Calibration Standards. The intact analysis was obtained in the positive linear mode (LP), applying an acceleration voltage of 25.09 kV, a grid voltage 45%, sample rate and digitizer setting 1.25 GS/s, lens 7.53 kV. LP spectra were calibrated externally with the trypsinogen, bovine albumin (BSA) protein and Bruker Bacterial Standard. The resulted mass spectra were acquired and processed using dedicated software, flexControl and flexAnalysis, respectively (both from Bruker Daltonics). Processed masses were submitted to MASCOT for protein identification. Peptide mass tolerance were set to 10 ppm.

3 Results and Discussion

The investigation shown describing and optimization of the environmental conditions having an impact on reproducibility and quality of one-dimensional intact-cell spectrometric approach and as well two-directional analysis of drugs and post-translation modifications (PTMs) of dairy proteins as like casein fraction, lactoferrin. It was discussed the use of MALDI technique in expression analysis of protein and nano-composite of metals. Moreover, the work presents potential peak markers of the analyzed bacterial strains and the results of off-line coupling of capillary electrophoresis of microbial aggregates with MALDI detection [4-6].

4 Conclusions

The MALDI-TOF (TOF)-MS technique is recommended mainly for biochemical and clinical analysis. The potential of MALDI-TOF/TOF-MS makes it possible to extend the applications to other fields of microbiological analysis, pharmacology, food technology or environmental analysis.

Acknowledgement

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DETERMINATION OF URINARY INDICAN AND CREATININE USING AN UHPLC/ECD-DAD METHOD

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Summary

Urinary indican (created from tryptophan) is excreted in the urine. Normally, only a small amount of indican is found in the urine. Higher concentrations are caused by intestinal dysbiosis. Hence, urinary indican is one of the most common and easily assessable markers of these complications.

A new UHPLC/ECD-DAD method was developed to analyze urinary indican and creatinine levels, especially in the urine of patients after intestinal surgery. This method can reveal, whether the gut is stitched well or not.

Creatinine is analyzed as a reference compound – measured indican concentration in human urine is related to the concentration of creatinine in the same urine sample.

1 Introduction

Urinary indican (indoxyl sulfate potassium salt) is in the human body created from tryptophan. The essential amino acid tryptophan is in the gastrointestinal tract converted to indole by intestinal bacteria. Indole is subsequently converted first to

indoxyl and then to urinary indican (see Fig. 1) which is excreted in urine. Normally, only a small amount of urinary indican is found in the urine. Higher concentrations point out intestinal dysbiosis, small intestine bacterial overgrowth, malabsorption states, gastric cancer and other similar complications [1-3].

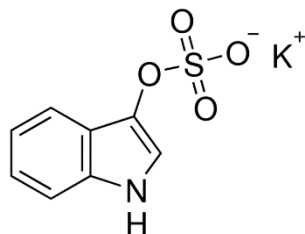


Fig. 1. The structure of urinary indican (indoxyl sulfate potassium salt).

Creatinine is analyzed as a reference compound – measured indican concentration in urine is always related to the concentration of creatinine in the same sample. It is utterly essential if you have just a one-time urine sample and not a 24-hour one [4].

2 Experimental

All chemicals, standards and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was deionized and purified in the IWA 20iol water purification system (Watek, Ledeċ nad Sázavou, CR).

The chromatographic UltiMate 3000 system consisted of pump model, autosampler, solvent rack, column compartment, electrochemical detector, diode array detector, pH monitor and conductivity monitor. Analytical column Agilent Zorbax SB-C18 250 x 4.6 mm, 5 μ m (Agilent Technologies, Santa Clara, CA, USA) was used.

Phosphate buffer (pH = 7.3) was used as the mobile phase (MP) A and methanol was used as MP B. The column was cooled to 20°C and the flow rate was set at 0.4 ml/min (5% B). Creatinine was spectrophotometrically detected at 500 nm and urinary indican was simultaneously electrochemically determined at +400 mV.

Pretreatment of urine samples included filtration and the Jaffe reaction – creatinine reacts with picric acid in an alkaline environment.

3 Results and Discussion

A new analytical method for quantity determination of urinary indican (related to concentration of creatinine in urine samples) was developed and validated.

Determination of creatinine itself was validated first. Afterwards, the same method was used for simultaneous determination of both creatinine and urinary indican. Fig. 2 shows chromatograms of these two compounds in a human urine sample. Both normal range of urinary indican and pathological range of this biomarker are presented.

This UHPLC/ECD-DAD method was evaluated by precision, accuracy, linearity, robustness, limit of detection and limit of quantification in a human urine matrix. Calibration solutions for determination of urinary indican and creatinine

concentrations can be prepared in ultrapure water. All results are in the form “ μmol of urinary indican/mmol of creatinine”.

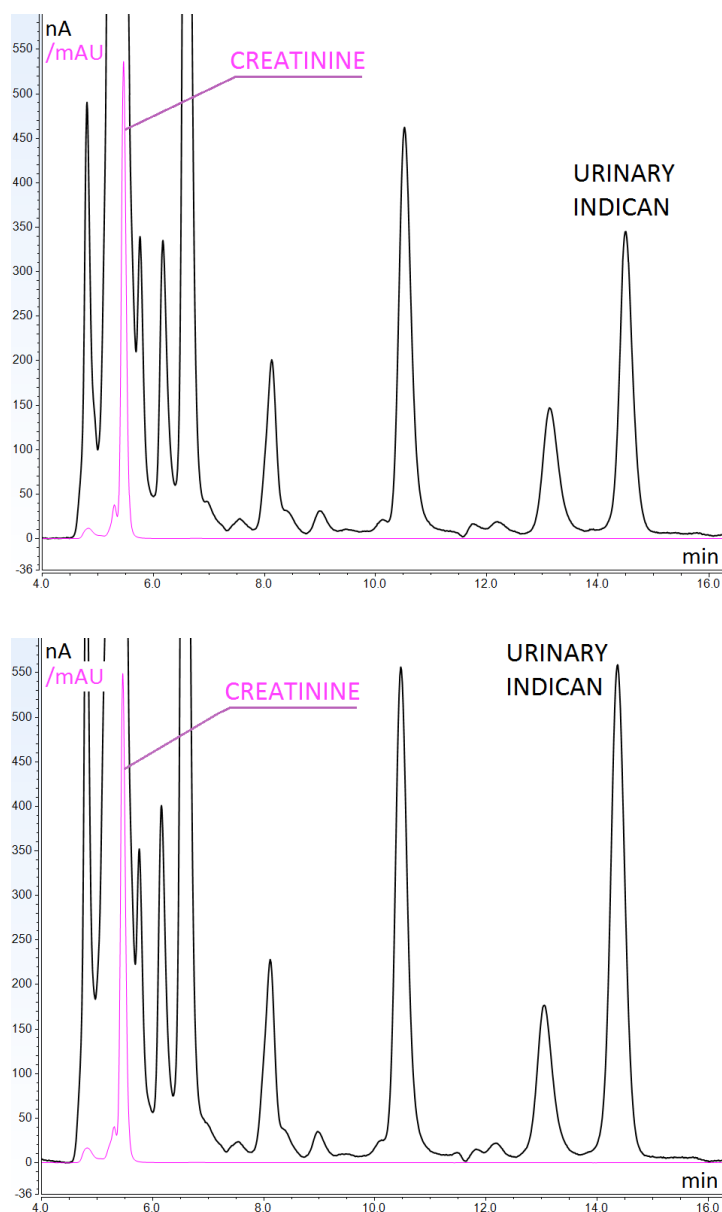


Fig. 2. UHPLC/ECD-DAD chromatograms of creatinine and urinary indican in a human urine sample – normal range (up) and pathological range of urinary indican (down).

4 Conclusions

It is possible to reveal higher concentrations of urinary indican and thus intestinal dysbiosis, small intestine bacterial overgrowth, malabsorption states, gastric cancer and other similar complications in the human body using the developed and validated

UHPLC/ECD-DAD method. Only a one-time urine sample is sufficient for this diagnostic test.

Acknowledgement

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BIO SILVER NANOPARTICLES AS A NEW OPPORTUNITY TO MEDICAL TREATMENT

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Summary

Development of a biologically inspired experimental process for synthesis of silver nanoparticles (AgNPs) is evolving into an important branch of nanotechnology. Applications in the field of medicine include the formulations of many potential antimicrobial agents, which are effective against many human pathogens including multidrug-resistant bacteria [1]. Multi-drug resistant strains of bacteria have become a serious problem in public health [2]. Therefore, the development of novel antimicrobial compounds or modification of available ones to combat resistant pathogens is urgently needed. However, it is well known that size of NPs influence the physicochemical properties and mechanisms of toxic action and in consequence the bioavailability [3]. A range of analytical techniques are available for providing information on particle size distributions, including microscopy approaches [4], chromatography [5], centrifugation [6], laser scattering [7], filtration [8], spectroscopic [9] and flow field flow fractionation [10]. The present work has been focused on the antimicrobial activity of metal bionanoparticles and their physicochemical characteristics using different techniques.

Acknowledgement

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SINGLE RUN GLYCAN STRUCTURE IDENTIFICATION FOR CAPILLARY ELECTROPHORESIS

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Summary

The use of capillary electrophoresis in biomedical research and the biopharmaceutical industry is facing tremendous growth, while the development of related data interpretation methods is lagging behind. In this presentation we report the design and implementation of a co-injected triple-internal standard method, to alleviate the need of

an accompanying run of the maltooligosaccharide ladder for glucose unit (GU) calculation. Based on the migration times of the co-injected standards of maltose, maltotriose and maltopentadecaose (bracketing the peaks of interest), a data processing approach was designed and developed to set up a virtual ladder that was used for GU calculation. The data processing was tested in terms of the calculated GU values of human IgG glycans and the resulting relative standard deviation was $\leq 1.07\%$. This approach readily supports high-throughput capillary electrophoresis systems by significantly speeding-up the processing time for glycan structural assignment.

1 Introduction

Glucose unit (GU) calculation plays prevalent role in database search based structural assignment. The GU approach is aiming to become system-independent, serving as a standard structural elucidation method in glycobiology. Despite its well-established role in the chromatography field, CE based data processing and interpretation is still in its early stages. In this presentation, a co-injected triple-internal standard method is utilized for instant glycan structure assignment, which does not require an associated maltooligosaccharide ladder run for GU value determination.

2 Experimental

The N-CHO Carbohydrate Analysis Kit (Sciex, Brea, CA), including all reagents necessary for glycan labeling and capillary electrophoresis separation was used in all experiments. 50 μm i.d bare fused silica (BFS) capillaries were from Polymicro Technologies (Phoenix, AZ). Human IgG and all other chemicals were purchased from Sigma Aldrich (St. Louis, MO). During the study, the capillary length, separation temperature, capillary type, and separation voltage were all altered as noted in the presentation. An inter-instrument study was also implemented (PA 800 Plus and MDQ systems).

3 Results and Discussion

Based on the migration times of the co-injected triple-internal standards, a virtual ladder was accurately calculated and used for rapid structural elucidation. The suggested approach was tested in terms of the resulted GU values for the major N-linked carbohydrates released from human IgG. It can conclude that the triple-internal standard method provided very accurate GU values without the necessity to run the maltooligosaccharide ladder before or after the sample analysis run. The highest calculated relative standard deviation was 1.07% which is well below the reported deviation values of NIBRT's GlycoBase [1]. With this novel utilization of the well-chosen three internal standards of maltose, maltotriose and maltopentadecaose, the method presented here provides accurate and reliable results even with data collected at a range of conditions (e.g. various capillary lengths, temperatures, coatings, separation voltages, and injection methods). Considering the fact that separation conditions should not influence the calculated GU values our method fulfills high-throughput requirements and should be of broad industrial and academic interest. In addition to serving as an internal GU standard, DP3 also acted as an internal labeling

standard providing information about the efficiency of the labeling reaction. Since the suggested method proved to be system-independent it overcomes the bottleneck of the traditional GU calculation approach, which was sensitive to experimental conditions.

Acknowledgement

This work was supported by the MTA-PE Translational Glycomics program (#97101) and the NKFIH K116263 grant of the Hungarian Government.

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SWITCHING OF PROTEIN PROPERTIES UPON GLYCATION REVEALED BY ELECTROCATALYTIC APPROACH

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Summary

In this contribution, a first sensing strategy for protein glycation is proposed, based on protein electroactivity measurement. Concretely, the label-free method proposed is based on the application of a constant-current chronopotentiometric stripping analysis at Hg-containing electrodes. The methylglyoxal-mediated glycation process was monitored as the decrease in the electrocatalytic protein signal, peak H, observed at highly negative potentials, which was previously ascribed to a catalytic hydrogen evolution reaction. Using this method, model water-soluble (bovine serum albumin, human serum albumin and lysozyme) and poorly water-soluble membrane (Na/K ATPase) proteins were investigated.

1 Introduction

Protein glycation is the result of the covalent bonding of the protein molecule with sugars and their metabolic by-products *via* a non-enzymatic process. The glycation of proteins occurs by a complex series of sequential and parallel reactions that form a Schiff's base, Amadori products and advanced glycation end-products (AGEs) [1].

In the physiological setting, glucose and other saccharides are important glycation agents, but the most reactive glycation agents are the α -oxoaldehydes, glyoxal, methylglyoxal and 3-deoxyglucosone. Methylglyoxal is the most significant glycation agent *in vivo*, being one of the most reactive dicarbonyl molecules in living cells. This compound is an unavoidable by-product of glycolysis. Methylglyoxal

irreversibly reacts with amino groups in lipids, nucleic acids and proteins, forming methylglyoxal-derived advanced glycation end-products (MAGEs) [1].

Protein glycation is a complex process that plays an important role in diabetes mellitus, aging and in the regulation of protein function in general. As a result, current methodological research on proteins is focused on the development of novel approaches for investigation of glycation processes.

2 Experimental

The samples (I, II and III) were analyzed using a.c. voltammetry (ACV) and constant-current stripping chronopotentiometric analysis (CPSA). Two types of working electrodes were used, *i.e.* hanging mercury drop electrode and silver solid amalgam electrode.

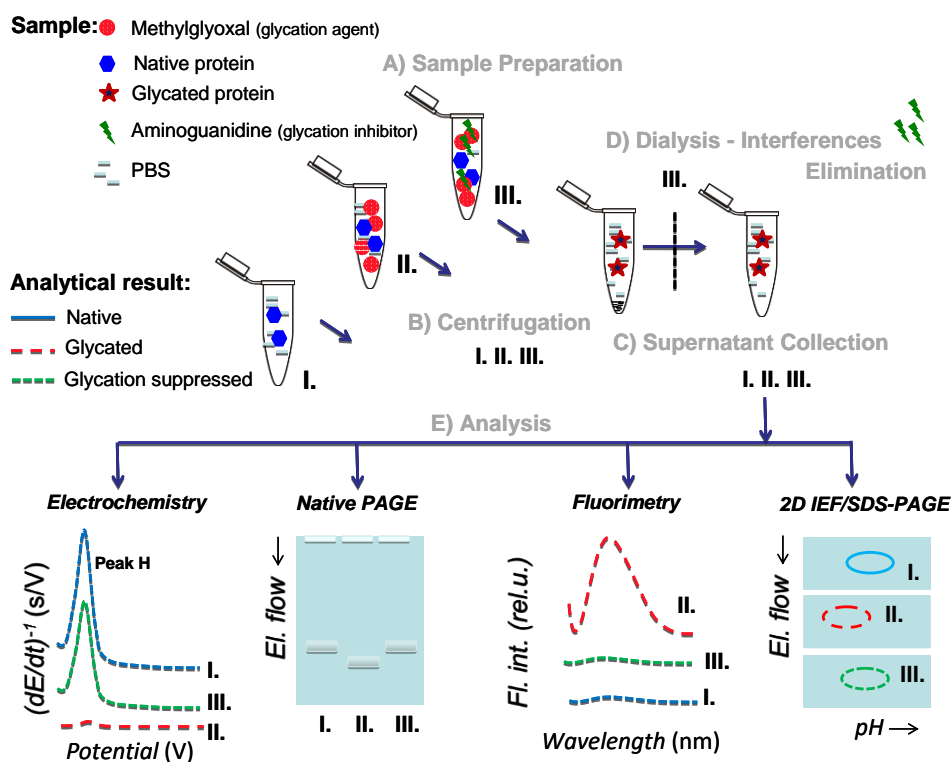


Fig. 1. Graphical representation of sample handling and analytical methods used for detecting protein glycation. Three sample types were investigated in this study: sample I (native protein), sample II (glycated protein), and sample III, where the glycation process was suppressed with the glycation inhibitor aminoguanidine (A). All samples were centrifuged (B), supernatants were collected (C), sample III was subjected to dialysis to eliminate interfering species (D), and finally the acquired supernatants (or dialyzates) were analyzed. CPSA was used for the analysis, and the results were compared with the results of complementary methods such as native PAGE, fluorescence spectroscopy and 2D isoelectric focusing/denaturing PAGE (E).

All measurements were performed at room temperature with a μ Autolab III analyzer (EcoChemie, NL) connected to a VA-Stand 663 (Metrohm, Herisau, Switzerland) in a three-electrode setup with Ag/AgCl3M KCl and Pt-wire as reference and auxiliary electrodes, respectively. For other details on experimental design and application of complementary methods see Fig. 1 [2].

3 Results and Discussion

Here we present a novel electrochemical label-free method for the *in vitro* monitoring of protein glycation *via* observing changes in intrinsic electroactivity of the protein. The study covers several model protein molecules and methylglyoxal as the glycation agent. The electrochemical results presented here are supported by previously developed complementary methods, *i.e.* native (PAGE) electrophoretic assay, 2D isoelectric focusing-polyacrylamide gel electrophoresis (SDS-PAGE) and the fluorescence spectroscopy method (Fig. 1) [2].

The general principle of the proposed assay is to monitor an electrocatalytic process called catalytic hydrogen evolution reaction (CHER), where the protein serves as the catalyst. The result of the measurement is an electrocatalytic CPS signal observable at negative potentials at Hg-electrodes known as peak H. Concretely, proton-donating amino acid (*aa*) residues, Cys and the basic *aa* residues Lys, Arg and His, are responsible for the electrocatalytic process, *i.e.* CHER [3].

In more detail, the principle of our approach is connected to the covalent modification (glycation) of the above-mentioned *aa* residues, because the submolecular targets for non-enzymatic protein glycation are primarily Lys, Arg, Cys and to a limited extent also His [1]. Thus, if the glycation reaction proceeds, the electrocatalytically active *aa* residues are not able to contribute to the CHER, which is reflected in the changes in peak H in the investigated protein samples.

4 Conclusions

Taking into account the fact that the glycation targets in proteins are the same *aa* residues as those participating in the electrocatalytic reaction, we are able to selectively monitor glycation processes in water-soluble [2] and also poorly water-soluble [4] proteins *via* the decrease in CPS peak H. The electrocatalytic approach applicability was demonstrated using analyses of real protein samples, which were isolated from blood of healthy volunteers.

Acknowledgement

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EXTRACTION, SEPARATION AND IDENTIFICATION OF BIOACTIVE COMPOUNDS IN BARLEY USING LC-MS/MS

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Summary

An extraction procedure, based on alkaline and acidic hydrolysis, was developed, using the *Box-Behnken* design of experiment, with the aim to achieve an exhaustive recovery of free and also linked polyphenols, i.e. bonded to cell structures, in barley (*Hordeum Vulgare L.*) samples. An LC-MS/MS (MRM transition mode) method was developed for analysis of the samples extracted through the optimized procedure, and to achieve a correct quali-quantitative characterization of a specific group of polyphenols.

The optimized procedure was also employed for the extraction and the identification of bioactive molecules in a dietary supplement composed of dried leaves of barley, commonly known as “young barley”. A *selectivity* study was carried out employing some of the compounds identified in young barley, and by using six different columns (C18, silica, ES-CN and OH5 types). The results showed that different mechanisms are involved in the retention of the standards, especially in case of HILIC mode.

1 Introduction

In the last years, the market of functional foods and dietary supplements has increased with a compound annual growth rate of 7%; consequently, the characterization of *nutraceuticals* is nowadays a topic of great interest. At present, polyphenols appear to be among the most interesting bioactive compounds, thanks to their extraordinary antioxidant properties, but still there is not a standard method for their extraction. However, in recent years, the researchers focused on the basic or acid, or both, hydrolysis process [1] in order to recover also linked polyphenols. Polyphenolic compounds, in fact, may be present in plants in free or esterified/etherified soluble form as well as in insoluble form, bound to the cell wall constituent. It is widely known that more than 90% of polyphenols in cereals are present in bonded form and are considered the major contributors to the total antioxidant capacity of cereals [2]. Therefore, for a

correct quali-quantitative characterization, these processes are required. Moreover, liquid chromatography-mass spectrometry (LC-MS) technique is nowadays the best analytical approach to study polyphenols from different biological matrices. On the basis of these knowledge, one of the aims of this research was to develop a suitable extraction procedure and a fast RPLC-MS/MS method, for the quali-quantitative characterization of barley.

Some polyphenols identified in a dietary supplement consisting of an extract of “young barley”, namely the plant in the first stage of growth (leaves), which is rich in minerals, vitamins and polyphenols [3], were chosen as standards to carry out a selectivity study. Separation selectivity is important fundamental parameter affecting resolution in LC, especially when the separation of complex mixture has to be achieved. The selectivity in various chromatographic modes strongly depends on types of interactions, and the combination of stationary and mobile phase is the key factor affecting the achieved results. The selectivity study performed enables comparison of different columns and stationary phases in order to select the best possible conditions, with the aim to develop a LC method suitable for the separation of all compounds.

2 Experimental

Box-Behnken design of experiment and *Statistica software version 12* (Stat Soft, Czech Republic) were used for the design and the evaluation of data, respectively, for the development of the optimized extraction procedure. LC-PDA analysis were performed using an HPLC system equipped with a binary gradient pump LC-20AD (Shimadzu), PDA detector (Shimadzu), column thermostat LCO 102 Single (Ecom). The LC-MS/MS analyses were performed using a Shimadzu modular liquid chromatograph consisting of two LC-20ADXR pumps, DGU-5 degassing unit, SIL-20ADXR autosampler and LCO-102 column thermostat (Ecom) coupled with QTRAP 4500 mass spectrometer operating in electrospray mode (AB SCIEX).

The column used was the Ascentis Express C18 (150 mm 3.0 mm, 2.7 μm). The injection volume was 1 μL , mobile phase consisted of HCCOH 0.4% (pH ~2.3) (solvent A) and CH_3CN (solvent B) and the step-wise gradient profile was as follows: 0 min, 12% B; 4 min, 12% B; 8 min, 30% B; 12 min, 70% B; 14 min, 12% B; 24 min, 12% B; the flow rate was 0.5 mL/min and the temperature was 30°C.

The column used for the selectivity study were the Ascentis® Express: HILIC (150 x 3 mm, 2.7 μm), ES-CN (100 x 2.1 mm, 2.7 μm), an OH5 (100 x 2.1 mm, 2.7 μm), a Kinetex® C18 (150 x 3 mm, 2.6 μm) and two Luna®: C18 (150 x 3 mm, 3 μm) and HILIC (150 x 3 mm, 3 μm). The standards used for characterization of barley and the solvents used for the optimization of the extraction procedure and the HPLC-PDA and LC-MS/MS analyses were purchased from Sigma-Aldrich. The standards used for characterization of young barley were purchased from Extrasynthese.

3 Results and Discussion

The parameters chosen for the extraction procedure were: 70% (v/v) of acetone, 100% of ethyl acetate and 25 min of sonication. With the first step of the procedure, only free

polyphenols can be recovered, so with the aim to have an exhaustive recovery, considering also the bound molecules, alkaline and acid hydrolyses were carried out, using ascorbic acid and EDTA to prevent eventual degradation phenomena. The extracted polyphenols of five different cultivars of barley were analyzed with the LC-MS/MS technique developed. Epicatechin was the only compound present exclusively in free form, while all the other have also be found after the basic and acid hydrolysis. Moreover, the presence of some polyphenols was observed only after the hydrolysis process, indicating that they are present only in bound form (catechin, myricetin, quercetin, kaempferol). These results are in agreement with the aim of this research, designed to establish the real need for a more complete extraction procedure, and for the correct evaluation of the phenolic content. The quali-quantitative composition of polyphenols presented in the five samples was quite similar, without significant differences. According to the literature data [4], *p*-hydroxybenzoic and ferulic acids were the most abundant, 624 $\mu\text{g/g}$ and 198 $\mu\text{g/g}$, respectively (average of the five cultivars). It's important to underline that, although *p*-hydroxybenzoic acid is the most abundant compound among the free molecules, its larger quantity was obtained only after the alkaline hydrolysis (sample β). Myricetin, quercetin and kaempferol are not present in free form, but they were detected after alkaline and acid hydrolysis. Apigenin exists in free and linked form, but it was found only in samples γ , in this case the basic hydrolysis was not enough to break the bonds, which link this molecule to some cell components.

Among the RP columns tested, the Ascentis Express C18 column provided the best resolution and the shortest time of analyses. The gradient was optimized using the *LC Simulator* software and further evaluated at different values of pH.

Using the LC-MS/MS method, it was found that young barley is rich in soluble polyphenols (linked to one or more molecules of sugar). Anyhow, some of them were found after hydrolysis procedure.

The columns tested to evaluate the retentions of different standards showed linear correlation between concentration of solvent B and $\log k$. However, in some cases, deviations from linearity were observed, demonstrating that there are many mechanisms involved in HILIC separation mode.

4 Conclusions

A new extraction procedure, aimed at the exhaustive recovery of free and also linked polyphenols was developed. The results showed the importance of the acidic and alkaline hydrolyses in this procedure. A HPLC-MS/MS method in MRM transition mode was developed for the correct quantitative characterization of polyphenols in barley. Young barley is rich in soluble polyphenols, while some of these compounds showed significant differences in retention, depending on the column used.

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DETERMINATION OF DISTRIBUTION CONSTANTS OF ANTIOXIDANTS BETWEEN LIPOSOMES, ALKYL SULFATE MICELLES, OCTANOL AND WATER USING ELECTROKINETIC CHROMATOGRAPHY

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Summary

The *in vivo* effects of the antioxidants are dependent on their lipophilicity, which govern membrane and protein interactions. Two electrokinetic systems, i.e. micellar electrokinetic chromatography (MEKC) and liposome electrokinetic chromatography (LEKC), were used for studying of the lipophilicity of antioxidants. Micelles of sodium decyl sulfate (SDeS) and sodium dodecyl sulfate (SDS) were used as the pseudostationary phase in MEKC and the liposomes were composed of mixtures of 1-palmitoyl-2-oleyl-*sn*-glycerophosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-phosphatidylserine (POPS) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidyl-DL-glycerol (POPG) in LEKC. The retention factors of the studied analytes were determined at pH 7.4. The micelle and liposome/aqueous distribution constants for studied antioxidants were experimentally determined and compared with values of octanol/aqueous distribution constants.

1 Introduction

Antioxidants are secondary metabolites of plants, which can protect organisms from the effect of free radicals. They are able to inhibit oxidative processes and improve the immune function [1]. Polyphenols, including flavonoids, are the most common natural antioxidants in a human diet. Flavonoids can be divided into six groups – flavones, flavonoles, flavanoles, flavanones, isoflavones, and anthocyanines. Phenolic acids can be divided into two groups based on the structure of cinnamic or benzoic acid. *In vivo* effects of these compounds are dependent on their lipophilicity and hydrophilicity. The logarithm of the octanol/water distribution constant, $\log P_{o/w}$, is the most common way of describing hydrophobicity of compounds. For charged compounds, logarithm of the distribution constant between octanol and aqueous solution with the target pH value is

used [3,4]. For better understanding of the interactions between lipophilic and hydrophilic phases, micelle and liposome/aqueous distribution constant (dependent on van der Waals and hydrogen donor/acceptor interactions) can be determined [5]. MEKC and LEKC were utilized for determination of the distribution constant between micelles, liposomes and aqueous phase at pH 7.4.

The distribution constant, K_D , is characterized by retention factor, k , and the phase ratio, Φ :

$$K_D = \frac{k}{\Phi} \quad (1)$$

2 Experimental

For LEKC and MEKC studies, the instrument for capillary electrophoresis Agilent 7100 (Agilent, Palo Alto, CA, USA) was used. The uncoated fused-silica capillary with 50 μm I.D. and total length of 36.5 cm (effective length 28 cm) was preconditioned by rinsing for 15 min with 0.1 M NaOH, 15 min with milliQ water, and 15 min (CZE, MEKC) or 5 min (LEKC) with the background electrolyte before the first run. The sample was injected by pressure 50 mbar for 5 s. The applied voltage was 20 kV, the temperature was 25 °C, and the wavelength was set at 214 and 254 nm. All CZE and MEKC experiments were repeated five times, whereas the LEKC experiments were repeated three times.

Phosphate buffer (ionic strength $I = 20$ mM and pH 7.4) was prepared by mixing the solutions of sodium hydrogen phosphate (purchased from Sigma-Aldrich, St. Louis, MO, USA) and sodium dihydrogen phosphate (from Mallinckrodt Baker, Deventer, The Netherlands). The phosphate buffer was filtered through a 0.45 μm filter. The BGE for MEKC contained SDS or SDeS (Sigma-Aldrich) in phosphate buffer ($I = 20$ mM) at pH 7.4. The BGE for LEKC was prepared from stock solutions of POPC, POPS, and POPG (all phospholipids were purchased from Avanti Polar Lipids, Alabaster, AL, USA) in chloroform (stored in a freezer), and phosphate buffer ($I = 20$ mM, pH 7.4). The concentration of the lipid dispersions, i.e., 80:20 mol% POPC/POPS and 80:20 mol% POPC/POPG, were 4 mM, which were diluted to 0.5 mM with phosphate buffer for LEKC analyses.

The concentration of the stock solution of each standard was 1 g/L in methanol (Mallinckrodt Baker, Deventer, The Netherlands). The concentrations of standards injected for analyses were 25 mg/L in milliQ water, each except for morin, biochanin A, esculin, isoquercitrin, luteolin, and myricetin, which were of 50 mg/L in water/methanol 3:1 (v/v). 0.5 mM thiourea (Sigma) in phosphate buffer was used as a marker of electroosmotic flow.

3 Results and Discussion

The mobilities of the analysed compounds were determined under CZE, MEKC and LEKC conditions. The electrophoretic mobilities of the micelles and liposomes were determined by iterative procedure using a homological series of alkylbenzoates (C1-

C6) [5-7]. The distribution constants of selected flavonoids and phenolic acids between liposomes (80:20 mol% POPC/POPS or 80:20 mol% POPC/POPG), micelles (SDeS and SDS) and phosphate buffer (I = 20 mM, pH 7.4) were determined. Obtained values were compared with literature values of octanol/aqueous distribution constants. The values of $\log D_{pH7.4}$ were used for ionized compounds and $\log P_{o/w}$ values for neutral compounds. $\log P_{o/w}$ values were predicted using the ACD/Labs Percepta Platform – PhysChem Module and the pK_a values were predicted by Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2015 ACD/Labs). Values of D were calculated by equation:

$$D = \frac{P_{o/w}}{1+10^{(pH-pK_A)}} \quad (2)$$

Based on the obtained results, the more polar compounds are only weakly retained in POPC/POPG and POPC/POPS liposomes. Significantly higher $\log K_D$ values were observed for some phenolic acids (i.e. gallic acid, 4-hydroxybenzoic acid, *p*-coumaric acid, and sinapic acid) when comparing POPC/POPG liposomes with POPC/POPS liposomes. This clearly indicates selective interactions between some of the compounds and the polar head groups of the lipids. Even though both lipids, i.e. POPG and POPS, have an overall negative surface charge, the polar parts of the molecules are dissimilar. POPS has two negatively charged functional groups and one neutralizing positively charged group, whereas POPG only contains one negatively charged phosphate group. The 1,2-diol in POPG might also result in specific interactions with the aforementioned compounds.

For comparison of the LEKC and MEKC separation systems and for characterization of the distribution equilibria, the correlation between the constants determined for SDS micelles and liposomes were evaluated. Both systems provided similar positive slopes of correlation with slightly better correlation of SDS with POPC/POPG, indicated by a higher Pearson's correlation coefficient (0.716 for SDS-POPC/POPG as compared to 0.661 for SDS-POPC/POPS).

4 Conclusions

In the work, CZE, MEKC and LEKC were used for the determination of the distribution constant of the selected flavonoids and phenolic acids. The pseudostationary phases in LEKC were formed by liposomes composed of 80:20 mol% POPC/POPS and 80:20 mol% POPC/POPG. Micelles of SDeS and SDS were used as a pseudostationary phase in MEKC. The obtained distribution constants for the compounds were compared with the literature values of *n*-octanol/aqueous phase. The results show that the distribution constants of phenolic acids and flavonoid glycosides are lower than the distribution constants of the flavonoids. The data showed stronger positive correlation between the two studied liposome systems than between the liposome-micelle system.

Acknowledgement

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BAL31-NGS IN TELOMERE BIOLOGY

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Summary

We combined a next generation sequencing approach and BAL31 digestion of high molecular weight genomic DNA for purposes of telomere sequence identification *de novo*. Using this method we identified two novel telomere motifs in plants lacking typical plant telomere sequences.

1 Introduction

A wide range of approaches was used for telomere sequence identification in model organisms, including protozoans, yeasts, animals and plants (reviewed in [1]). Each of the models has specific advantages and features. The number of telomere sequential motifs is still increasing and the big picture of telomere motif evolution becomes high resolution image. There are some rare exceptions (e.g. Diptera with telomere retrotransposons), but mostly these telomere motifs are derived from the consensus $T_xA_yG_z$ tandem repeat. Variability, exceptions and unknown telomeres open doors of advanced knowledge, which can be potentially used in biotechnologies, agriculture and medicine. Our effort was to develop method based on a next generation sequencing (NGS) approach for telomere sequence identification in large plant genomes *de novo* (ca. 2 Gb per chromosome).

2 Experimental

In the NGS-BAL31 approach, based on the two most general features of eukaryotic telomeres, their repetitive character and sensitivity to BAL31 nuclease digestion, we have used advantage of the capacity and current affordability of NGS, in combination with the robustness of classical BAL31 nuclease digestion of chromosomal termini. While representative samples of most repeat elements were ensured by low-coverage (even less than 5%) and relatively low-cost genomic shot-gun NGS, candidate telomeres from BAL31 treated samples were identified as under-represented sequences. The bioinformatics was done in two parallel ways using either RepeatExplorer pipeline (RE) or Tandem Repeats Finder (TRFi). [2, 3] In case of TRFi we also used custom made scripts for data post-processing [1].

3 Results and Discussion

The NGS-BAL31 method was tested on two groups of ornamental and economically important plants: i) dicots, genus *Cestrum*, and ii) monocots, *Allium* species (e.g. *A. ursinum* and *A. cepa*). Both groups of plants have species with large genomes (tens of Gb) and a low number of chromosomes ($2n=14-16$). Their genomes are enlarged by repeats. Both plant genera lack typical telomere sequences and multiple studies have attempted to characterize them unsuccessfully. However, despite some results using classical molecular biology approaches, interesting hypotheses and suggestions of alternative candidate telomeres (retrotransposons, rDNA, satellite repeats), these studies have not resolved the question. We have identified telomere sequences in both tested plant groups using NGS-BAL31 and confirmed their real telomere role by conventional molecular biology tests as FISH, telomerase assay and terminal restriction fragments analysis [4, 5].

4 Conclusions

NGS is a common tool in research and diagnostics. NGS data will become a commonly used source of information about telomere sequences in model organisms, less explored species, species with unknown telomeres sequences and very probably clinical samples. We have shown that under-representation of telomeres in NGS data after BAL31 treatment is a simple and feasible way how to characterize telomere sequences even in large genomes with a limited number of chromosomal ends. However, BAL31-NGS should presumably work on smaller genomes too. We recommend using RE and TRFi analysis in parallel for telomere sequence identification *de novo*. Both tools are genome reference and assembly or any repeat database independent. Beyond our original intention, BAL31-NGS can be applied in combination with other well established bioinformatics approaches like peak finding software, such as the Bayesian algorithms employed by ChIP-seq analysis. This combination can achieve nice progress and accuracy in proximal/distal localization of telomere motif variants or non-telomeric sequences hired to telomeres in ALT positive organisms and tumor cells [6, 7].

Acknowledgement

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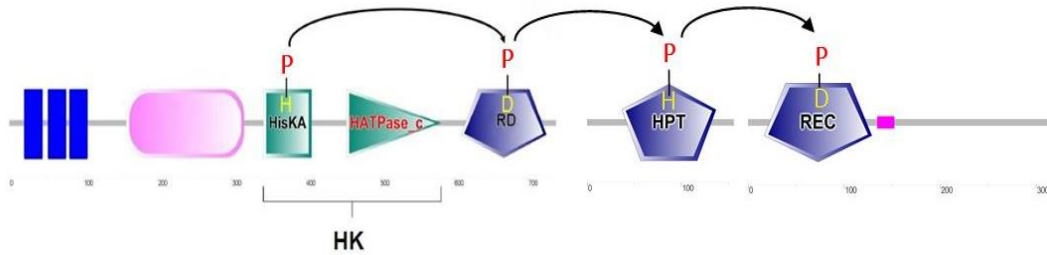
ROLE OF *IN VITRO* PHOSPHORYLATION ASSAY IN UNDERSTANDING MSP SIGNALING IN *A. THALIANA*

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1 Introduction

Multistep phosphorelay (MSP) is a backbone signaling pathway in plants and is based on protein (trans) phosphorylation. MSP consists of the hybrid homodimeric sensor histidine kinase (HK), a His-containing phosphotransfer (HPt) domain and a response regulator (RR). Transphosphorylation occurs intramolecularly from a phosphorylated histidine of the catalytic domain of HK to a conserved aspartate of the C-terminal receiver domain (RD). The HPt protein accepts the signal and transfers it to the RD of RRs in nuclei, which either induce transcriptional changes or interact with effector proteins [1]. The MSP activity can be monitored *in vitro* by an assay called transphosphorylation which results in the incorporation of radiolabeled phosphate from [γ - ^{32}P] ATP into a peptide or protein substrate [2]. Our recent work provides evidence for a crosstalk between ethylene and cytokinin signalling (Zdarska et al., manuscript in preparation). In the present study, mechanistic insight into ethylene signal transduction via MSP pathway in *A. thaliana* is demonstrated by means of this assay.



2 Experimental

HK domain of ethylene receptor 1 (ETR1) from ethylene signaling pathway, C-terminal RD of CYTOKININ-INDEPENDENT 1 (CKI1) and HPT domain AHP2 from cytokinin signaling pathway were tested. CKI1_{RD} was also mutated in catalytic aspartate as well as at sites D1057G, G1058N. Purified, cytoplasmic, soluble ETR1_{HK} as the first acceptor of phosphate was autophosphorylated with 32 nM [γ -³²P]-ATP in a kinase buffer for 1 h at room temperature, then split into separate reactions containing equivalent amounts of each purified RDs and AHP2. Each reaction was incubated for an identical period of time and then stopped by addition of sample buffer, separated by SDS-PAGE, and imaged on phosphor screens [3].

3 Results and Discussion

Transfer of a phosphate group from ETR1_{HK} domain to CKI1_{RD} and then to AHP2 was observed. It is an evidence of interaction between ETR1 protein and MSP. The highest intensity of phosphate signal appeared in 40 mins. The mutations of the active site aspartate (D1050A and D1050E) suppressed the CKI1_{RD} activity and transfer to AHP2. Other mutations (D1057G, G1058N) located in β 3- α 3 loop gave the same results. This loop follows the active site D1050 in the crystal structure and helps to achieve the conformation of CKI1_{RD} allowing its activation via phosphorylation [4]. The loop β 3- α 3 CKI1_{RD} is highly flexible and the mutations in it change its dynamics resulting in no phosphate binding (Otrusínová et.al. submitted).

4 Conclusions

CKI1_{RD} is non-cognate receiver domain for ETR1_{HK} in its wild form and catalytic aspartate is crucial for its activity. The results obtained so far indicate that cross-talk between the MSP and ethylene signaling can take place via C-terminal RD of MSP histidine kinase.

Acknowledgements

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A NOVEL RAPID METHOD FOR DETECTING PROTEIN-NUCLEIC ACID INTERACTIONS

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Summary

We have decided to employ protein induced fluorescence enhancement (PIFE) to develop readily available method to study protein/NA interactions. Here we demonstrate on BamHI, Ku and XPF/ERCC1 proteins the versatility and applicability of this method using microwell plate fluorescence readers (mwPIFE).

1 Introduction

Many fundamental biological processes depend on intricate networks of interactions between proteins and nucleic acids and quantitative description of these interactions is important for understanding mechanisms that govern transcription, DNA replication or translation. PIFE represents a phenomenon whereby protein increases fluorescence of a cyanine dye when it binds to its vicinity. PIFE has been mainly used in single molecule studies to detect protein associations with DNA or RNA. Here we present a novel application of PIFE for steady state quantification protein/nucleic acid interactions.

2 Experimental

Biotinylated dsDNA oligonucleotides labeled with Cy3 dye were immobilized onto a Neutravidin coated plate. Proteins BamHI, Ku, XPF/ERCC1 were used in this study and added to the DNA. Protein enhancement was calculated from fluorescence prior and post protein addition and normalized to signal obtained in the absence of the protein.

3 Results and Discussion

We demonstrate on the examples of the restriction enzyme BamHI, and DNA repair complexes Ku and XPF/ERCC1 general applicability of mwPIFE in examining various aspects of protein/DNA interactions. These include determination of sequence and DNA structure binding specificities, dissociation constants, detection of weak interactions and protein ability to translocate along DNA.

4 Conclusions

mwPIFE represent relatively easy and high throughput method that does not require protein labeling and can be applied to a wide range applications involving protein/NA interactions.

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CAPILLARY ELECTROPHORESIS WITH ONLINE NANORAECTOR FOR CHARACTERISATION OF SKIN FIBROBLAST ELASTASE ACTIVITY FROM VARIOUS BIOLOGIC MATERIAL

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Summary

In this study we were focused on the determination of skin fibroblast elastase (SFE) activity in different biologic material by capillary electrophoresis using nanoreactor based on mixing by transverse diffusion of laminar flow profiles. SFE is one of the important enzyme involved in skin aging because it causes reduction of elastin fibers. We examined activity of commercially available purified enzyme and cells lysate prepared from human normal fibroblasts and keratinocytes. Finally the effect of well known inhibitors of SFE – LD-Thiorphan and oil from the nuts of *Calophyllum inophyllum*, locally called “Tamanu oil” known for its beneficial impact on the skin, was evaluated.

1 Introduction

Skin fibroblast elastase (SFE) is in the literature also known as neprilysin, (NEP; EC 3.4.24.11), neutral endopeptidase 24.11, enkephalinase, membrane metallo-endopeptidase and is also identical to common acute lymphoblastic leukemia antigen or CD10 [1, 2]. General function of this enzyme is the reduction of cellular response to peptide hormone, therefore SFE has various roles in organism, e.g. cleavage of enkephalines in brain, dissociation of peptide modulating blood pressure and degradation of amyloid β peptide in brain. Role of this enzyme in skin aging is

participation on the degradation of the elastic fibers which form a network that contributes to the elasticity and resilience and their decreasing results in wrinkle formation. Discovering of new SFE inhibitors can be promising for all previously mentioned fields.

In this study the 5-carboxyfluorescein labeled peptides were used as substrates. Their SFE cleavage resulted in fluorescently labeled products, which enabled to employ very specific and also sensitive LIF detection, in contrast to common assay with substrate *N*-succinyl-tri-alanyl-*p*-nitroaniline using UV detection. All incubations in this study were performed by online nanoreactor to minimize reactant consumption.

2 Experimental

2.1 Cell lysis

For cell lysate preparation, protocol was adapted from [3]. Briefly, cells were detached with trypsin 1X, and collected by centrifugation 10 min at 1500 rpm. Cells pellets were resuspended in lysis buffer, and incubated on ice 20 min. Soluble proteins were then collected in supernatant after centrifugation 10 min at 12000 rpm.

2.2 On-line capillary electrophoresis

Conditions for the capillary electrophoresis assay from previously published method [4] were adapted in this study. Analyses were performed in a PA800+ instrument from AB Sciex (Brea, CA, USA) equipped with laser induced fluorescent detector (λ_{ex} 488 nm, λ_{em} 520 nm) with a capillary of 50 μm internal diameter and 60 cm total length. The effective detection length was 10.2 cm. The capillary temperature was maintained at 30°C during all steps. The background electrolyte consisting of 50 mM tetraborate buffer (pH 8.7) was used. Reactants dissolved in incubation buffer (IB) 25 mM HEPES, 20 mM NaCl and 10 mM NaOH were injected into the capillary in sequence: IB (0.3 psi, 15s); enzymatic solution (0.5 psi, 4s); examined compound solution (0.5 psi, 4s); substrate (0.5 psi, 4s); examined compound solution (0.5 psi, 4s); enzymatic solution (0.5 psi, 4s); IB (0.5 psi, 20s) and incubated for 5 min. Separation was accomplished by the application of a voltage – 12 kV.

3 Results and Discussion

Lysis of cell can be realized by multiple ways e.g. using sonication, mechanical homogenization, difference in ionic strength of buffers or by detergents. In this study, different detergents were tried and optimization of lysis agent for cell lysis was conducted. The influence of various SDS and Triton X-100 concentrations on the activity of commercially available SFE was tested. Ionic detergent – SDS caused denaturation of enzyme and loss of activity to the contrary of outcome with non-ionic detergent – Triton X-100. As a result the cell lysate was prepared with the optimal concentration of Triton X-100 and the kinetic parameters for two labeled substrates were successfully determined.

Thereafter well know selective inhibitor of SFE, LD-Thiorphan, was used to verify the presence of enzyme in cell lysate. As it can be seen, the inhibition profiles of purified SFE and lysate were almost identical, see Figure 1. For further verification the

cell lysate of another cell types - keratinocytes was used which does not contain SFE and so the activity of SFE in this material against both substrates was negligible.

Finally the effects of LD-Thiorphan and Tamanu oil applied directly on cells in two concentration levels were studied. After 24 hours of incubation with examined compounds, the cells were collected, counted and lysed by protocol described above. LD-Thiorphan did not show any significant effect on the activity of the investigated enzyme in cells after 24 hours of exposure. It can be assumed that LD-Thiorphan did not cause any changes in expression of SFE and also did not modulate SFE activity.

The effect of Tamanu oil applied directly on fibroblasts cells was described recently by group of Antsel [5]. They observed increased cell viability and accelerated wound healing after Tamanu oil application on cells. In this study we found increased activity of SFE in cells affected by Tamanu oil. It can be caused by modification of activity of SFE or increased expression of this enzyme in the fibroblasts. Determination of activity enzyme participating on production of elastin –lysyl oxidase that catalyzes formation of cross-linking of elastin, can provide a complete description of the situation.

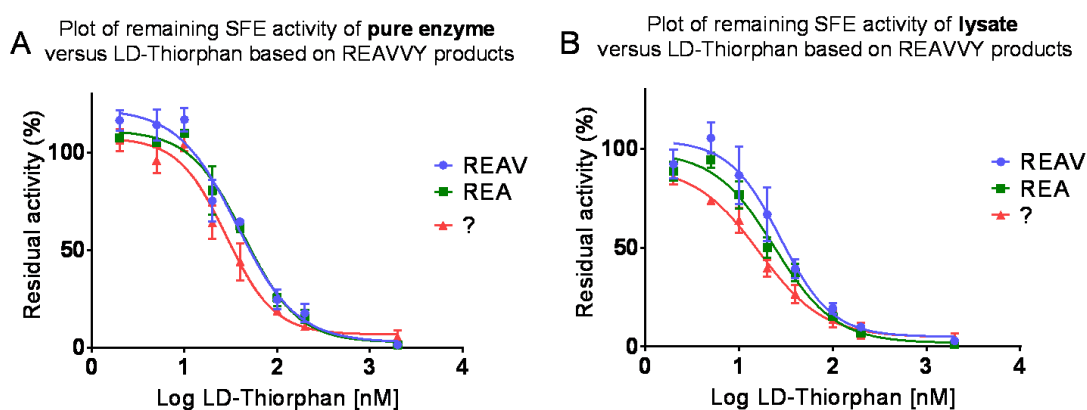


Fig. 1. Plots for remaining SFE activity of pure enzyme (A) and lysate (B) after inhibition by LD-Thiorphan.

4 Conclusions

In this study we present utilization of in-capillary online reaction of labeled substrates with SFE. LIF detection of resulting products offers high sensitivity and excellent selectivity. Enzyme activities of commercial purified enzyme and lysate prepared from different cell types were compared to verify the nature of obtained enzyme activity in cell lysates. Besides the effect of LD-Thiorphan and Tamanu oil on fibroblasts SFE activity was evaluated.

Acknowledgement

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Abstracts of poster presentations

P1 SEPARATION OF SOME 1,4-DIHYDROPYRIDINE DERIVATIVES BY MICELLAR ELECTROKINETIC CHROMATOGRAPHY

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Summary

Micellar electrokinetic chromatography method for lipophilicity determination of some 1,4-dihydropyridine derivatives have been developed. Electrophoretic behaviour of these compounds which possess self-assembling properties and spontaneously form nanoaggregates (liposomes) in an aqueous environment was investigated in various background electrolytes. It was found that the most suitable environment for their analysis was 50 mM borate buffer with 50 mM SDS and 30 % propan-1-ol.

1 Introduction

Dihydropyridines (DHPs) represent a large group of structurally diverse compounds based on a pyridine core. Although DHPs were primarily developed as cardiovascular agents, several have been used for other medical applications because they have a broad range of pharmacological actions as agents in vasodilation, antiatherosclerosis, antidiabetes or antitumor agents [1]. Moreover, cationic amphiphiles synthesized on the basis of polyfunctional 1,4-dihydropyridine (1,4-DHP) derivatives possess self-assembling properties and spontaneously form liposomes in aqueous environments [2]. In this work, we used micellar electrokinetic chromatography (MEKC) to separate derivatives of 1,4-DHPs.

2 Experimental

All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) or Acros Organics (New Jersey, USA). Derivatives of 1,4-DHP were synthesized and obtained from the Latvian Institute of Organic Synthesis (Laboratory of Membrane active Compounds and β -diketones). All the experiments were performed on capillary electrophoresis HP ^{3D}CE with DAD detector (Agilent Technologies, Waldbronn, Germany).

3 Results and Discussion

First, we studied electrophoretic behaviour of neutral and positively charged 1,4-DHPs in various background electrolytes with different pH values and ionic strength. Also influence of presence of various organic solvents (acetonitrile, methanol, ethanol, propan-1-ol and butan-1-ol) and concentration of them (5 – 40 %, v/v) in separation buffers and samples were investigated. The effect of organic additive is crucial for obtaining the separation. It was found, that most suitable environment for analysis of neutral and charged 1,4-DHPs is 50 mM borate pH 9.5 with 50 mM SDS and 30 % propan-1-ol.

4 Conclusions

In this work, we have developed MEKC method for determination of some 1,4-DHP derivatives. This method will be used for determination of lipophilicity (log P values) of biologically active compounds and for study of self-assembly effects including nanoparticles.

Acknowledgement

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P2 MALDI MS IMAGING WITH SCANNING LASER BEAM

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Summary

Demand for high-throughput matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) mass spectrometry imaging (MSI) is growing with the tendency towards

increasing the lateral imaging resolution to the range of micrometers. Rapid MALDI MSI can be achieved by movement of the laser beam across the sample surface using an optical scanner, producing discrete pixels.[1] In this contribution, we present development of a high-throughput MALDI TOF instrumentation with scanning laser beam. Modification of an ion source for efficient laser beam scanning and also design of a high-repetition rate circuitry for pulsed extraction and construction of an ion mirror are discussed. The system performance is demonstrated on imaging of model sublimated samples as well as on 3D cell aggregates of melanoma cancer cells. Acquisition rates $>50 \text{ pixel.s}^{-1}$ were achieved using the laboratory-built MALDI TOF MS instrument.

1 Introduction

TOF analyzers offer spectra acquisition significantly faster compared to the other mass analyzers and as such are predestined to the fast MSI analysis. MSI speed limitations of the MALDI TOF systems equipped with kHz lasers are dependent on the employed sampling mode and capabilities of computing system processing and storing a vast amount of generated data. In the traditional sampling mode, stage translations ($\sim 100 \mu\text{s}$) between individual measuring points present a major contribution to the total MSI time. The movement of the laser beam ($<1 \mu\text{s}$) can, to some extent, substitute for the stage translation in one axis and allows truly rapid MSI.

2 Experimental

2.1 Laboratory-built MALDI TOF

A high-throughput TOF mass spectrometer was equipped with a 355-nm 5-kHz Nd:YAG laser and a galvanometer based optical scanner redirecting the laser beam on a sub-millisecond time scale. The ion source featured two grid ion electrodes ($500 \mu\text{m}$ mesh) for ion extraction/acceleration and two motorized linear stages for sample positioning. Two wires in the extraction grid were excised making a path for a scanning laser beam. A dual stage ion mirror with the total length of $\sim 470 \text{ mm}$ was mounted at the end of the 2.6 m long flight tube. A pulser with two identical MOSFET switches allowed high voltage pulsing for delayed extraction at the 5 kHz repetition rate. Instrument control software was optimized for parallel data acquisition, processing and storage.

2.2 Model sample preparation

The samples were prepared using GPE-1207-030PS glass sublimation apparatus (GPE Scientific Ltd, Bedfordshire, United Kingdom). Amount of 0.3 g of DHB was spread at the bottom of the apparatus and sublimed at $120 \text{ }^\circ\text{C}$ and 60 mTorr resulting in approximate 4 – 5 μm thick layer of DHB on target. Plain stainless steel targets and targets with sections of the gelatin embedded 3D melanoma cell culture aggregates (spheroids) treated with cancerostatics were used.

3 Results and Discussion

3.1 Ion source design

The scheme of the ion source is shown at Fig.1. The creation of the small window in the ion extraction electrode mesh allowed scanning of the laser beam across the sample in the range of $\sim 1\ 200\ \mu\text{m}$ without laser beam being blocked by the grid wires. The incident angle 20° allows generation of almost circular laser spot profile at the target. The design of the laser beam optics allowed focusing the laser beam down to $10\ \mu\text{m}$, which is suitable for MSI with high lateral resolution.

Ion source modification did not deteriorate the mass resolving power ($R = 20\ 000$ at $2\ 465\ \text{Da}$) of the instrument nor affected the instrument sensitivity.

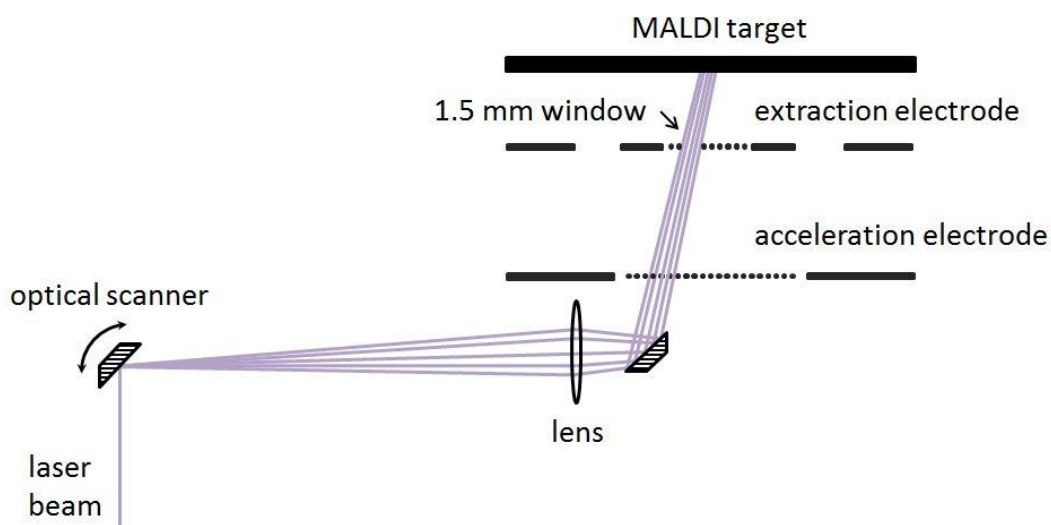


Fig. 1. Scheme of the ion source arrangement showing laser beam focused through ion source electrodes.

3.2 MSI speed

The performance of the system will be discussed in detail as a function of spot size, pixel size, laser fluence, number of data points in spectrum etc. The speed enhancement is highest at low pixel size and high laser repetition rate, which allows efficient employment of the laser beam scanning mode. For example, $10\ 000$ -pixel image acquired at 5-kHz repetition rate, 20 shots per $20\text{-}\mu\text{m}$ pixel, $100\ 000$ pts per spectrum, is recorded in about 3 minutes with acquisition rate $>50\ \text{pixel}\cdot\text{s}^{-1}$.

3.3 MSI of model samples and spheroids

Model sublimated DHB layer was used to optimize MSI parameters, which were subsequently used for imaging of spheroids. Spheroids were imaged at 5-kHz repetition rate using 1-mm laser beam scanning with 20 shots per $20\text{-}\mu\text{m}$ pixel. Fine structure of the spheroids and gelatin was revealed proving the effectiveness of the developed

instrumentation. Lipids and selected cancerostatics were also identified in the spheroid tissues.

4 Conclusions

The laboratory-built MALDI TOF MS was upgraded to allow rapid laser beam scanning MSI at 5-kHz laser repetition rates. The modified ion source with grid electrodes was introduced for the laser beam scanning MSI. A single galvanometer-based optical scanner redirecting the laser beam across the sample surface in range ~1mm allowed high-resolution MALDI TOF MSI with acquisition rates $>50 \text{ spectra.s}^{-1}$. The MS images of 3D melanoma cell aggregates were generated revealing distribution of the cancerostatics and lipid species.

Acknowledgement

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P3 IDENTIFICATION OF DIFFERENT ISOMERS OF SHORT-CHAIN ACYLCARNITINES BY HPLC-ESI-IT-TOF-MS

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Summary

The main goal of this work was to develop an analytical method for identification of different isomers of short-chain acylcarnitines in dried blood samples (DBS) from newborns by using high-performance liquid chromatography with tandem mass spectrometry (HPLC-ESI-IT-TOF-MS). The chromatographic separation was performed on ACQUITY UPLC[®] HSS C18 column with a gradient elution by using a mobile phase A (10 mmol.L⁻¹ formic acid) and mobile phase B (acetonitrile). The mass spectrometer was operated in a positive ionization mode with the mass range 100-900 m/z.

1 Introduction

Short-chain acyl-CoA dehydrogenase (SCAD) deficiency (SCADD) is an autosomal recessive inborn error of the mitochondrial fatty acid oxidation with highly variable clinical, biochemical and genetic characteristics. This belongs to a group of diseases known as fatty acid oxidation disorders, which are the most common inborn errors of metabolism. Mitochondrial beta oxidation of fatty acids is an essential energy producing pathway [1].

SCADD is biochemically characterized by increasing the concentration of C4 carnitine in the blood. Four-carbon acylcarnitine exists as two isomers: butyrylcarnitine and isobutyrylcarnitine. The plasma concentration of the first one is increased in patients with short-chain acyl-CoA dehydrogenase (SCAD) deficiency, and the second one is increased in those with isobutyryl-CoA dehydrogenase (IBD) deficiency. However, both these genetic defects are associated with an increased C4- carnitine signal, and are indistinguishable by conventional screening methods [2].

Many methods have been developed to determine acylcarnitines, and the majority of them use mass spectrometry (MS). Mass spectrometry is a commonly used technique in routine assays, such as in neonatal screening, however, limitations to this method are reported (acylcarnitine constitutional isomers cannot be distinguished). This type of analysis is typically performed by selecting the appropriate precursor ion which products ions for all analytes in the single run. Despite its simplicity and great success for the use in newborn screening, it has no ability to resolve isomers and can give false positive results [3]. Therefore, in order to separate isomers, liquid chromatography (HPLC) is used as a separation method before the mass spectrometric detection. However, samples have to be processed with a tedious derivatization pretreatment using butanol or other synthesized reagents because the hydrophilic carnitine and short-chain acylcarnitines have a poor retention on a reverse phase column [4].

2 Experimental

Chemicals used for the MS measurements were LC-MS purity, water (LiChrosolv Hypergrade) was purchased from Merck (Darmstadt, Germany), acetonitrile was from Fisher Scientific (Loughborough, UK) and formic acid was from Sigma-Aldrich (Steinheim, Germany). Derivatized dried blood samples from newborns and control levels of DBS were obtained from Newborn Screening Centre of Slovak Republic, Children Faculty Hospital Banská Bystrica. Standards of butyryl and isobutyryl carnitine were purchased from Sigma-Aldrich.

HPLC-MS/MS analyses were performed by using Shimadzu LCMS-IT-TOF™ analyzer (Shimadzu, Kyoto, Japan). The used mass spectrometer combines the electrospray ionization (ESI), ion trap (IT) and time-of-flight (TOF) mass analyzer, which enable it to perform MSⁿ experiments. Data acquisition and processing were performed using Shimadzu LCMS Solution software v.3.51. During MS and MS/MS measurements, the mass spectrometer was used in the positive ionization mode depending on the analyzed compounds in the scanning range 100-900 m/z.

Chromatographic separations were performed on ACQUITY UPLC® HSS C18 (50 x 2.1 mm; 1.8 μm) (Waters, Ireland), with the column temperature set to 40° C. The

mobile phase A consisted of 10 mmol.L⁻¹ formic acid in water and mobile phase B was acetonitrile, with the gradient profile 10 – 90 % B and total analysis time 10 min. The flow rate of mobile phase was 0.2 mL.min⁻¹. The injection volume was 5 µL.

3 Results and discussion

This work was focused on the separation and identification of two different isomers of C4 carnitine (butyryl- and isobutyryl carnitine) in the complex biological matrix such as a dried human blood spot using HPLC-ESI-IT-TOF-MS. DBS and control levels L1, L2 at two different concentrations of acylcarnitines were derivatized by commercial reagent kit from Chromsystems, stored at 4°C and were reconstituted just before HPLC-ESI-IT-TOF-MS analyses. First part of our work was focused on the optimization of chromatographic and mass spectrometric conditions in order to achieve an optimal ionization of carnitines and separation of both isomers. Optimal conditions are described in section “Experimental”. Fig.1 shows TIC and XIC records of C4 carnitine butyl esters, obtained after HPLC-ESI-IT-TOF-MS analysis of DBS from a healthy newborn. As can be seen from this record, C4 carnitine butyl esters create two peaks with a low intensity for the ion with m/z 288.2184, what is in agreement with literature sources [3, 4].

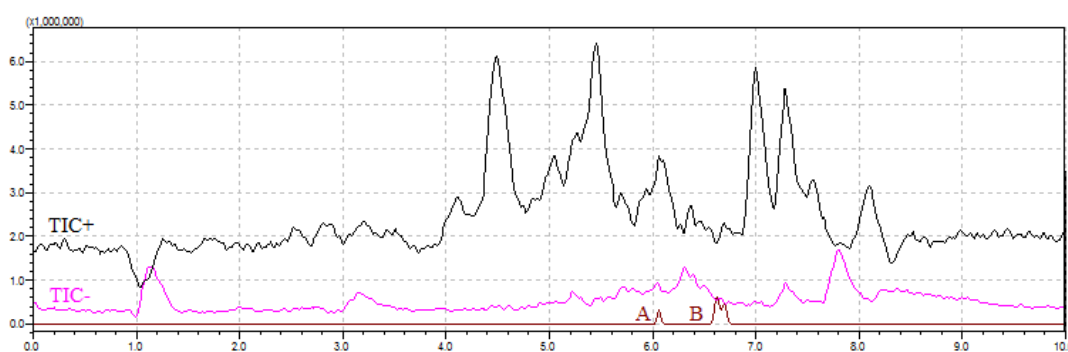


Fig. 1. TIC and XIC records of C4 carnitine obtained after HPLC-ESI-IT-TOF-MS analysis of DBS from a healthy newborn, A - isobutyrylcarnitine, B - butyrylcarnitine.

Fig. 2 shows the TIC and XIC records of C4 carnitine obtained after HPLC-ESI-IT-TOF-MS analysis of DBS from a newborn with an elevated amount of C4 carnitine. On this record, two peaks corresponding to a molecular ion of butyryl- and isobutyrylcarnitine with a higher intensity than was obtained for a healthy newborn, are clearly visible. The HPLC-ESI-IT-TOF-MS identification of butyrylcarnitine and isobutyrylcarnitine was confirmed against standards.

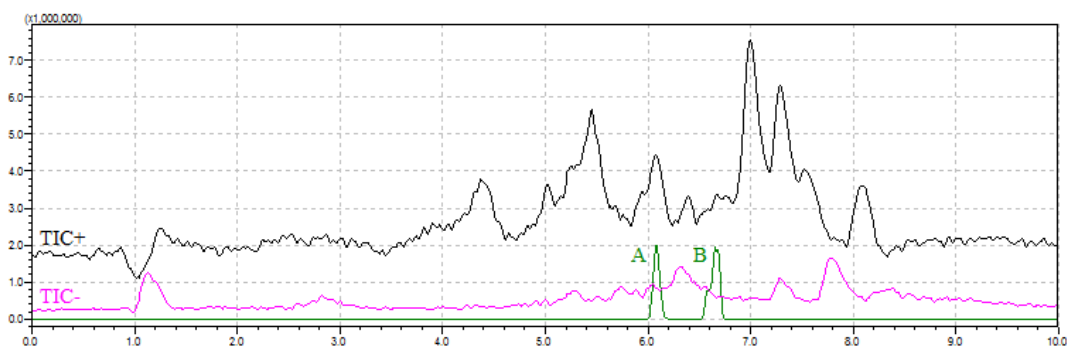


Fig. 2 TIC and XIC records of C4 carnitine obtained after HPLC-ESI-IT-TOF-MS analysis of DBS from a newborn with mitochondrial fatty acids oxidation disorders (elevated amount of C4 carnitine), A - isobutyrylcarnitine, B - butyrylcarnitine.

4 Conclusion

In this work, a simple, fast and sensitive HPLC-ESI-IT-TOF-MS method for the separation and identification of two isomers of C4 carnitine was developed. The proposed method was tested on individual C4 carnitine standards as well as on dried blood samples obtained from healthy newborns and patients with mitochondrial fatty acids oxidation disorders.

Acknowledgement

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P4 NITROGEN DOPED ORGANOSILICON PLASMA POLYMERS FOR BIOAPPLICATIONS

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Summary

The aim of the present work was to prepare organosilicon thin films with amine functionalities on single crystalline silicon substrates using plasma enhanced chemical

vapor deposition (PECVD). The film properties prospective for bioapplications (surface free energy, resistance against mechanical damage, chemical composition, i.e. the presence of primary and secondary amines) were studied.

1 Introduction

Plasma polymerized hexamethyldisiloxane (pp-HMDSO) coatings have been attracting interest of many researches mainly due to the possibility to vary their properties in a very wide range. These materials have a great potential to succeed in large field of applications such as anti-scratch layers for plastics, corrosion protection films, coatings for pharmaceutical packaging etc. The functionalization of organosilicon plasma polymers has been investigated for development of unique biomaterials. The aim of our work was to add a suitable doping agent during plasma polymerization and to form specific functionalities tailoring chemistry, physical properties as well as biocompatibility of the films [1, 2].

2 Experimental

The studied thin films were deposited from mixtures of HMDSO ($\text{SiO}_2\text{C}_6\text{H}_{18}$), nitrogen and methane applying RF glow discharges at low pressure ($10 \div 30$ Pa) in a parallel plate reactor. The bottom electrode served as the substrate holder and it was coupled to RF generator (13.56 MHz) via a blocking capacitor. Double-side polished silicon substrates were placed on the bottom electrode, the RF voltage of which was superimposed with a negative DC self-bias. The supplied power was kept at 50 W for all depositions. The flow rate of HMDSO Q_H was in the range from 0.3 to 0.4 sccm, the methane flow rate was in the range from 0 to 1.4 sccm and the nitrogen flow rate Q_N ranged from 1 to 10 sccm. The coatings chemical composition was investigated by Fourier transform infrared spectroscopy. The chemical composition was investigated by X-ray photoelectron spectroscopy (XPS). The surface free energy (SFE) was measured using contact angle measurements (See System, Advex Instruments).

3 Results and Discussion

The surface free energy of the prepared films increased with increasing nitrogen flow rate from 37 to 43 mJ/m². The film hardness and elastic modulus had the opposite tendency, they decreased with Q_N from 9 to 0.7 GPa and from 65 to 18 GPa, respectively. The XPS study of surface chemistry showed presence of Si, O, C, N. Oxygen is the most abundant element on surfaces of analyzed organosilicon polymers. Its atomic concentration varies between 49-60 at. % in dependence on gaseous mixture used. Films with maximum quantity of oxygen consist of minimum amount of nitrogen and carbon. Atomic concentration of silicon was ~35 at. %.

The high amount of oxygen possibly arises from the high amount of adsorbed water, which was found in absorption peaks in measured IR spectra around 1600 cm⁻¹ and 3400 cm⁻¹, which are mostly created by OH vibrations.

4 Conclusions

Organosilicon thin films with nitrogen-containing functional groups were deposited using RF glow discharges. The films were investigated by several methods aimed primarily at determination of chemical composition, surface microstructure and mechanical properties.

Acknowledgement

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P5 SCREENING OF ACYCLOVIR PERMEATION THROUGH SKIN USING ALAPTIDE

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Summary

This investigation deals with the affection of the permeation of acyclovir through full-thickness pig ear skin using a Franz diffusion cell from the donor vehicle of propylene glycol/water (1:1) using synthesised (*S*)-8-methyl-6,9-diazaspiro[4.5]decane-7,10-dione, alaptide as a potential transdermal permeation enhancer.

1 Introduction

Transdermal therapeutic systems are drug delivery systems designed for systemic administration. Due to their pharmacokinetic advantages they are a good alternative to traditional formulations with systemic administration. To reach the blood capillaries and achieve systemic therapeutic effect, a drug must be able to cross the skin barrier, especially the outermost layer, *stratum corneum*. Unfortunately, transdermal drug delivery often faces the problem of insufficient or no permeation of drug substances through the skin. The use of chemical permeation enhancers (CPEs) is one of the approaches for facilitating drug delivery through the skin – modification (i.e. hydration,

lipid fluidization and/or disruption) of *stratum corneum* [1]. Small polar molecules containing a characteristic fragment of heteroatoms X-CO-N=, where X is -CH₂-, -NH₂ or -NH-, may break the intermolecular H-bonds that hold the ceramides together in the *stratum corneum* [2]. One of CPEs, (S)-8-methyl-6,9-diazaspiro[4.5]decane-7,10-dione, known by the INN of “alaptide“ (see Fig. 1) is an original Czech compound prepared in the 1980s [3]. This compound demonstrated the excellent enhancement activity in a number of *in vitro* tests. Acyclovir (see Fig. 1) is an antiviral drug from the class of antimetabolites. Acyclovir is suitable for topical and systemic therapy [4]. Since it is poorly water-soluble and has poor oral bioavailability we investigated the effect of alaptide as a potential CPE on the permeation of acyclovir substance through the skin.

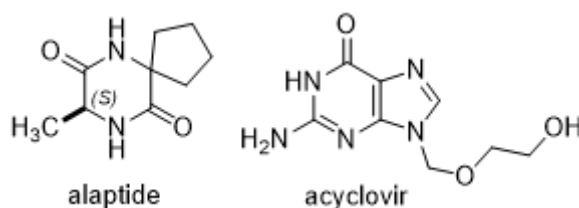


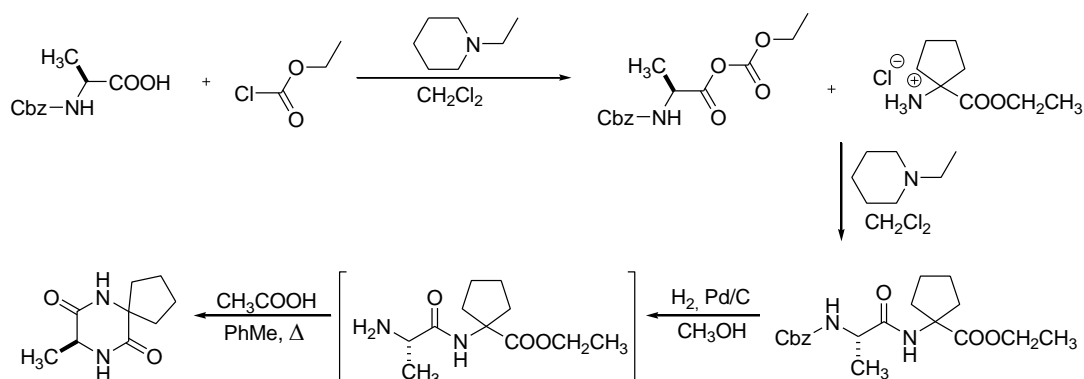
Fig. 1. Structures of alaptide and acyclovir.

2 Experimental

In vitro skin permeation experiments were performed using static Franz diffusion cells [5] (SES – Analytical Systems, Germany) with a donor surface area of 0.6359 cm² and a receptor volume of 5.2 mL within 24 hours. Full-thickness pig ear skin was selected for *in vitro* evaluation of permeation. This tissue is a suitable *in vitro* model of human skin [6, 7], because porcine skin has shown to be histologically and biochemically similar to human skin [8]. The permeation of acyclovir through the skin without and with alaptide (ALA) was tested from the donor vehicle of propylene glycol/water (1:1). The HPLC analysis of the samples was performed using an Agilent 1200 series HPLC system, equipped with a diode array detection (DAD) system. A Gemini C₆-Phenyl 110A 5 μm, 250×4.6 mm (Phenomenex®, USA) chromatographic column was used. The total flow of the column was 1.0 mL/min; injection was 10 μL; column temperature was 40 °C. The detection wavelength of 254 nm was chosen, the time of analysis was 7 min. A mixture of acetonitrile (HPLC grade, 5.0 %) and H₂O (HPLC – Mili-Q Grade, 95.0 %) was used as a mobile phase. The retention time (t_R) of acyclovir was 4.9±0.05 min; the limit of detection (LOD) was 0.0031 μg/mL; and the limit of quantification (LOQ) was 0.0104 μg/mL. The values obtained from the permeation experiments were expressed as the cumulative permeated amount of the drug (Q_t [μg]) per unit of skin surface area [9], see Table 1. The dependences of the cumulative permeated amount of the drug per unit of skin surface area in time are illustrated in Fig. 2 that is divided into two parts for better lucidity.

3 Results and Discussion

(*S*)-8-methyl-6,9-diazaspiro[4.5]decane-7,10-dione originally developed by *Kasafirek* [10] was prepared according to the scheme from protected L-alanine and ethyl ester of cycloleucine. Due to the lack of active chromophore the process of synthesis was extensively optimized with help of mass spectrometry.



Scheme 1. Synthesis of (*S*)-8-methyl-6,9-diazaspiro[4.5]decane-7,10-dione

From the *in vitro* skin permeation experiments is clear, that permeated amount of acyclovir with ALA increased rapidly already at the 30th minute, since without ALA acyclovir was not determined. The permeated amount of acyclovir with ALA at the 60th minute reached acyclovir 4-fold higher values than formulation without ALA, see Table 1. It can be stated that acyclovir without ALA permeated moderately in comparison with acyclovir with added ALA; in the whole investigated time range in every time the corresponding Q_t values related to the system without and with ALA were statistically different from each other, see Fig. 2.

Table 1. Cumulative permeated amounts Q_t per unit area [$\mu\text{g}/\text{cm}^2$] of acyclovir (ACL) from propylene glycol:water (1:1) without and with ALA as potential CPE achieved in *in vitro* transdermal permeation experiments using Franz diffusion cell. Q_t values are expressed as mean \pm SD ($n = 5$ experiments).

Time [h]	Cumulative permeated amounts Q_t per unit area [$\mu\text{g}/\text{cm}^2$]	
	ACL	ACL+ALA
0.5	0.0 \pm 0.0	0.6 \pm 0.1
1.0	1.3 \pm 0.4	5.0 \pm 0.4
1.5	3.4 \pm 0.4	11.2 \pm 1.0
2.0	7.1 \pm 0.7	20.6 \pm 2.2
3.0	17.2 \pm 0.6	41.3 \pm 2.9
4.0	33.3 \pm 3.0	71.1 \pm 2.5
6.0	65.0 \pm 5.8	126.4 \pm 5.0
8.0	105.5 \pm 5.8	197.0 \pm 5.0
12.0	199.0 \pm 5.5	348.5 \pm 6.1
24.0	523.7 \pm 17.2	823.3 \pm 6.4

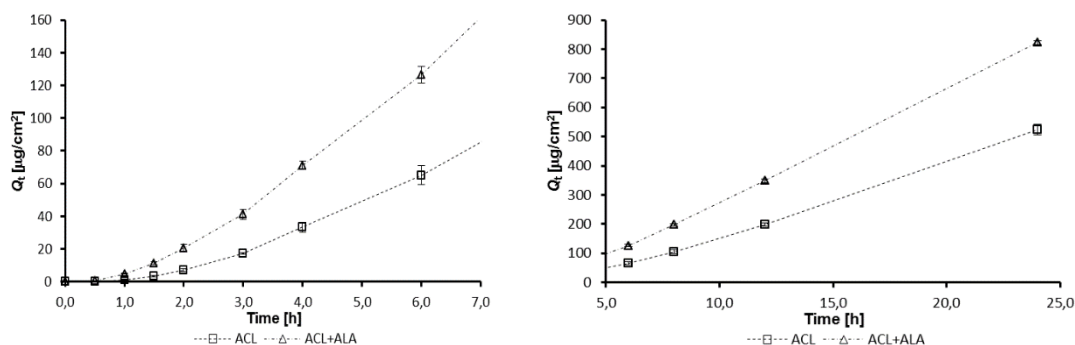


Fig. 2. In vitro profile of cumulative permeated amounts Q_t per unit area [$\mu\text{g}/\text{cm}^2$] in time of ACL alone and after addition of ALA in ratio 10:1 (ACL:ALA) from propylene glycol/water (1:1) system through skin. Q_t values are expressed as mean \pm SD ($n = 5$ experiments).

4 Conclusions

(*S*)-8-methyl-6,9-diazaspiro[4.5]decane-7,10-dione, alaptide (ALA) was prepared by optimized synthesis in 76% overall yield. The ability of ALA to enhance the penetration of acyclovir was examined. It can be assumed that the contribution of ALA to the enhanced permeation of acyclovir through the skin is significant not only immediately after application but also for long-term application.

Acknowledgement

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P6 **COMPUTER ASSISTED DESIGN OF A CONTINUOUS FLOW
CELL CULTURING MICRODEVICE**

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Summary

Design and feasibility study of a microfluidic cell culture device is described. Polydimethylsiloxane (PDMS) is known as a suitable material to fabricate microfluidic devices due to its advantageous properties. In addition, it can be used for cell culture vessel prototyping [1]. Real-time optical monitoring with a CCD camera facilitates the accurate observation of the cell or tissue population growth [2]. A microfabricated continuous flow cell culture vessel was designed using computational fluid dynamics simulations. The developed microfluidic cell culture device was fabricated using a conventional soft lithography method and tested with HT29 cancer cells.

1 Introduction

Microfluidic devices are attractive platforms for molecular biology and diagnostic applications. The use of microchips significantly decrease the volumes of fluids required. Subculturing cell cultures is a widely used method in microbiology, but rather labor intensive and time consuming task. Implementation of cell culturing in a lab-on-a-chip system has numerous advantages, such as reduced reagents consumption and labour-intensity. Furthermore, online observation of the breeding cells serves valuable information about population growth.

PDMS is a frequently applied polymer for fabrication of microfluidic devices, because of its advantages properties, i.e. biocompatibility, easy patterning at micrometer scale, strong bonding ability to glass substrate, relatively high chemical resistivity and low manufacturing cost.

2 Experimental

The microfluidic systems were manufactured by soft lithography technique using PDMS polymer and applying SU-8 epoxy based photoresist as molding replica. The surface of the PDMS channels was air plasma treated using our in-house developed microwave plasma reactor at 500 W for 5 seconds in vacuum. It helps to activate the surface of the PDMS and bond to a glass carrier surface. Firstly, the microchannels were treated with 5% 3-aminopropyltriethoxysilane (APTES, Sigma-Aldrich, St Louis,

MO) - ethanol solution for 1 hour. The APTES-coated surface was then activated with 2% glutaraldehyde (AppliChem Lifescience, Darmstadt, Germany) for 1 hour. Next, the activated surface was incubated with protein G (Sigma-Aldrich) at a concentration of 200 $\mu\text{g ml}^{-1}$ in PBS overnight at 4 °C. The excess silanol sites were blocked with bovine serum albumin (BSA, Sigma-Aldrich) solution (2 mg ml^{-1} in PBS) for 15 minutes. EpCAM mouse antibody (Life Technologies, Carlsbad, CA) at a concentration of 6 $\mu\text{g ml}^{-1}$ in PBS was then added to the protein G layer and incubated for 3 hours. After these functionalization steps the microchannels were washed with PBS. The EpCAM antibody functionalized microchips were tested with HT29 colon cancer cell suspension at a concentration of 20000 cells/mL. (National Institute of Oncology, Budapest, Hungary).

3 Results and Discussion

A microfabricated continuous flow cell culture vessel was designed based on computational fluid dynamics simulations in order to reduce development time and costs. Furthermore, online optical detection system was also developed to observe cell growth in the microfabricated continuous flow device. The system comprised a microscope objective coupled with CCD camera, the developed continuous flow cell culturing device, a UV LED source and notebook for data.

Acknowledgement

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**P7 DETERMINATION OF DICARBOXYLIC ACIDS IN
ATMOSPHERIC AEROSOLS USING ION CHROMATOGRAPHY
WITH ON-LINE CONNECTED PRECONCENTRATION UNIT**

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Summary

A sensitive analysis of dicarboxylic acids (DCAs) in atmospheric aerosols is presented. The whole system consists of four specific parts, namely the water-based condensation growth tube (GT), the aerosol collector (ACTJU), preconcentration system and ion chromatography. All this parts are on-line connected and works fully automated for continuous measuring with 30 min intervals. Oxalic, malonic, succinic, glutaric, adipic, pimelic, azelaic and maleic acid were selected as compounds of interest. Besides of this DCAs, we also monitored pyruvic and glyoxalic acids and common anions such as fluorides, chlorides, nitrites, nitrates, sulphates and phosphates. Limits of detection of DCAs are in the range of concentrations 10–30 ng.m³.

1 Introduction

Atmospheric aerosols are responsible for a number of deleterious effects on human and environment. Detailed study of chemical composition of atmospheric aerosols is important for both their role in various environmental issues and correlation with adverse health effects.

Atmospheric aerosols are composed of many organic compounds that originate from various primary sources or are formed secondary in the atmosphere from other oxidation of gas-phase precursors.

Dicarboxylic acids are group of compounds of both primary (traffic or biogenic emissions) and secondary (photochemical oxidation / decomposition) origin. DCAs in atmospheric aerosol have received much recent attention because of their capabilities to form CCN and affect thus the global radiation balance and eventually the global climate [1].

2 Experimental

Measurement of chemical composition of aerosols is presently performed mostly by sampling of aerosols at filter-packs or impactors with subsequent off-line analysis. Alternatively, on-line aerosol collectors based on a condensation of steam on particles are used [2,3,4].

Another approach is applied in aerosol collector called Aerosol Counter-flow Two Jets Unit (ACTJU), where a deionized water at room temperature instead of steam is employed for the continuous sampling of atmospheric aerosol particles [5].

3 Results and Discussion

The original version of ACTJU collector under optimum conditions collects quantitatively aerosol particles down to $0.3\ \mu\text{m}$ in diameter while the collection efficiency of smaller particles decreases. A combination of original version of ACTJU collector with a water-based condensation growth tube (GT) located upstream of the ACTJU sampler [6] allows quantitative sampling of aerosol particles down to a few nm in diameter. The collector effluent is permanently sucked out from the ACTJU for subsequent on-line analysis of particulate water-soluble species.

The combination of the GT/ACTJU collector with preconcentration column and online connected ion chromatography (ICS 2100, Dionex) allows direct sampling and analysis of atmospheric aerosol. The collector effluent is permanently sucked out from the GT/ACTJU to the preconcentration column and in the next step is via valve injected to the separation column of ion chromatography. Chromatogram of DCAs is shown in Fig. 2.

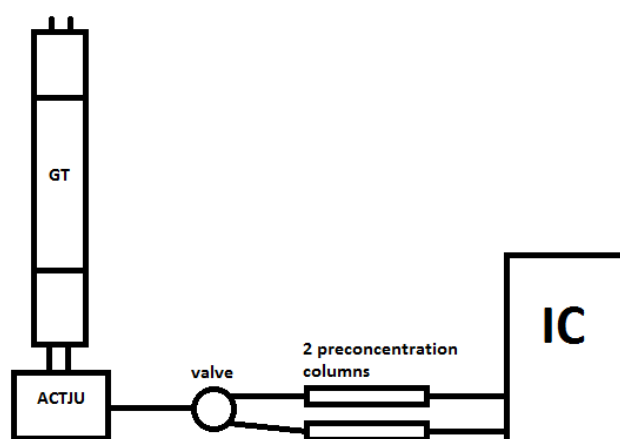


Fig. 1. Scheme of apparatus.

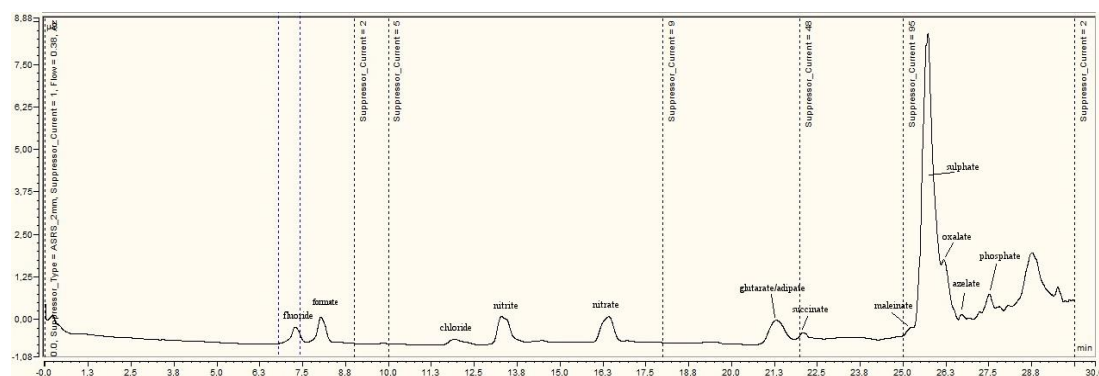


Fig. 2. Chromatogram of real sample of urban aerosol (Brno, June 2016).

4 Conclusions

A method for the fast and sensitive detection of dicarboxylic acids in atmospheric aerosols was optimized. The method is based on the online sampling and subsequent analysis with ion chromatography. The system was applied to the determination of DCAs in urban aerosols in Brno. The method was verified using a laboratory generated standard aerosol of DCAs. A sampling of aerosol particles on parallel filter was used as a reference method.

Acknowledgement

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**P8 COMPARISON OF ON-CAPILLARY DERIVATIZATION OF
AMINO ACIDS USING TDLFP AND EMMA MIXING OF REACTANTS FOR
CAPILLARY ELECTROPHORESIS COUPLED WITH LIF DETECTION**

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Summary

Amino acids play significant role in living organisms. Capillary electrophoresis combined with laser induced fluorescence detection is a powerful and sensitive analytical technique suitable for determination of amino acids after their derivatization. Derivatization reaction can be done pre-capillary, on-capillary or post-capillary. Mixing of the reactants on-capillary can be performed by longitudinal diffusion, by transverse diffusion of laminar flow profiles, or by electrophoretically mediated microanalysis. Automatic on-capillary derivatization of amino acids using naphthalene-2,3-dicarboxaldehyde/sodium cyanide as derivatization reagents were mixed either by transverse diffusion or electrophoretically. Both mixing approaches were individually optimized and eventually compared.

1 Introduction

Amino acids (AAs) are very important compounds for living organisms. They exhibit low or no native fluorescence, thus derivatization is essential for their sensitive determination by laser induced fluorescence (LIF) detection. Capillary electrophoresis (CE) offers conducting the derivatization reaction in pre-capillary, on-capillary or post-capillary arrangement. In the case of on-capillary derivatization, mixing of the reactants can be performed by longitudinal diffusion, by transverse diffusion of laminar flow profiles (TDLFP), or by electrophoretically mediated microanalysis (EMMA) [1].

Derivatization reagent naphthalene-2,3-dicarboxaldehyde (NDA) used in this work reacts with primary amines in the presence of nucleophile, such as cyanide anion, to form fluorescent 2-substituted 1-cyanobenz[*f*]isoindoles (CBIs) [2]. This paper deals with optimization of EMMA for simultaneous derivatization of all standard AAs. Previously published on-capillary derivatization of AAs using TDLFP methodology [3] was compared with optimized EMMA with respect to efficiency of mixing of reactants, sensitivity, repeatability and applicability to various biological samples.

2 Experimental

2.1 Model sample

Model sample of 21 AAs was used for optimization of separation and derivatization conditions. It was composed of 5 μM of Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Ser, Tau, Thr, Trp, Tyr, Val, 15 μM Ala-Gln and 20 μM Cyn and Lys diluted in the reaction buffer (100 mM $\text{H}_3\text{BO}_3/\text{NaOH}$, pH 9.8) containing 2 μM nVal

(internal standard, IS). Aliquots of 2× concentrated model sample were stored frozen at -20 °C and thawed at room temperature at day of the use.

2.2 Method

The Agilent G7100A CE System (Agilent Technologies, Waldbronn, Germany) coupled with external ZETALIF™ LED 480 detector (Picometrics, Labège, France) using λ_{ex} 480 nm and λ_{em} 515 nm was utilized in this study. The photomultiplier tube voltage was 600 V and rise time was set to 0.5 second. Background electrolyte (BGE) was composed of 135 mM H₃BO₃/NaOH, 73 mM sodium dodecyl sulfate (SDS), pH 9.00, 0.5 mM hydroxypropyl- β -cyclodextrin (HP- β -CD), 6.7 % (v/v) 1 propanol. BGE was filtered using nylon membrane filter with 0.45 μm porosity and degassed in an ultrasonic bath for 5 minutes. Fused silica capillary of 66.0 cm total length, 45.0 cm effective length and 50/375 μm inner/outer diameters was used. Capillary was kept at 25 °C and separation voltage was +30 kV.

3 Results and Discussion

3.1 EMMA derivatization

Difficult optimization is a general drawback of EMMA; however, the starting parameters can be obtained thanks to knowledge of the separation parameters such as separation voltage, total length of the capillary, injected plug lengths and electrophoretic mobilities of the reactants. Electrophoretic mobilities of AAs were taken from PeakMaster database [4]. Optimization of EMMA mixing of the reactants comprised of optimization of lengths of injected plugs of the reactants, time and the voltage of the electrophoretic mixing ensuring ideal overlap of the injected plugs, pH of the reaction buffer, concentrations of NDA and NaCN, and time of reaction. Because parameters of the electrophoretic mixing depend on the lengths of injected plugs and electrophoretic mobility of the reactants and vice versa, these parameters needed to be optimized cyclically. Optimized reaction conditions for EMMA comprise of sequential pressurized injection of 1 mM NDA in the reaction buffer with 12.5 % MeOH, sample in the reaction buffer and 1 mM NDA by pressure of 50 mbar for 24, 7 and 8 s, respectively. Finally, 2.5 mM NaCN in the reaction buffer was injected by pressure of 50 mbar for 34 s followed by electrophoretic mixing of the reactants by voltage of -5 kV for 65 s. The purpose of hydrodynamic injection of plug of NaCN was to keep injected plugs in the capillary, which are pushed out by electroosmotic flow during the electrophoretic mixing. The reaction was performed for 3 minutes at 25 °C. Total length of injected plugs was responsible for lowering the separation efficiency, thus pre concentration by sweeping was applied. Sweeping plug comprised of 100 mM SDS dissolved in stock solution of reaction buffer (200 mM H₃BO₃/NaOH, pH 9.8) was injected prior injection of the reactants by pressure of 50 mbar for 60 s. When the separation voltage (+30 kV) was applied, AA-CBIs interacting with SDS micelles were swept towards the capillary inlet.

3.2 TDLFP derivatization

In order to compare on-capillary derivatization method using EMMA, the previously published mixing of reactants by TDLFP using the three-zone arrangement was also performed [3]. Briefly, it comprises of sequential pressurized injection of 1.5 mM NDA in the reaction buffer with 50 % MeOH, sample in the reaction buffer and 10 mM NaCN in the reaction buffer (each plug 25 mbar for 6 s). Laminar flow was induced by injection of BGE by pressure of 25 mbar for 30 s. The reaction was conducted for 5 minutes at 25 °C.

3.3 Comparison of TDLFP and EMMA approach

Fig. 1 shows the electropherograms of the model sample derivatized by TDLFP (Fig. 1A) and EMMA (Fig. 1B). The electropherograms are depicted in the same scale. The peak heights in the electropherograms clearly shows higher degree of conversion most likely originating in increased efficiency of the mixing in the case of EMMA. Moreover, EMMA is evidently less prone to varying of the reaction yield by the sample composition. Both derivatization methods were also compared in terms of sensitivity and repeatability.

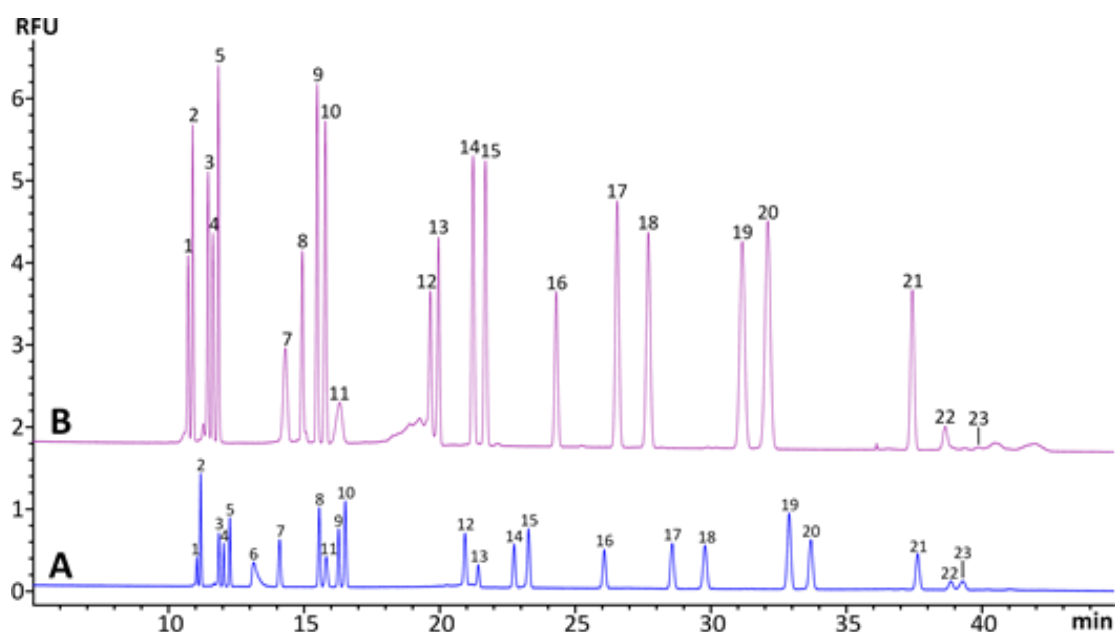


Fig. 1. The electropherograms of AA-CBIs of the model sample using TDLFP (A) and EMMA (B) approach. The electropherograms are depicted in the same scale. A: Sequential injections: NDA, model sample, NaCN by pressure of 25 mbar for 6 s each zone; reaction time: 5 min. B: Sequential injections: NDA, model sample, NDA and NaCN by pressure of 50 mbar for 24, 7, 8 and 34 s, respectively; electrophoretic mixing -5 kV 65 s, reaction time: 3 min. BGE: 135 mM H₃BO₃/NaOH, 73 mM SDS, pH 9.00, 0.5 mM HP- β -CD, 6.7 % (v/v) 1 propanol; capillary: 50/375 μ m inner/outer diameters, 66/45 cm total/effective length; voltage: +30 kV; temperature: 25 °C; detector: ZETALIF™ LED 480, λ_{ex} 480 nm, λ_{em} 515 nm, photomultiplier tube voltage 600 V, rise time 0.5 s. Peak numbering of AA CBIs: Asn (1), Ser (2), Gln (3), Thr (4), His (5),

Cyn-mono-CBI (6), Glu (7), Gly (8), Tyr (9), Ala (10), Asp (11), Tau (12), Ala-Gln (13), Val (14), Met (15), nVal (16), Trp (17), Ile (18), Leu (19), Phe (20), Arg (21), Cyn-bis-CBI (22) and Lys (23).

4 Conclusions

Two in principle different approaches for on-capillary mixing of reactants, namely EMMA and TDLFP, were compared. The EMMA based method combined with sweeping pre-concentration, provides on average 5.3× higher sensitivity (2.1-13.1×), comparable precision in terms of migration times, but on average 2× better precision in peak areas. Finally, results obtained by EMMA are significantly less dependent on the sample matrix.

Acknowledgement

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P9 OPTIMIZATION OF GRADIENT SEPARATION CONDITIONS IN TWO-DIMENSIONAL LIQUID PHASE SYSTEMS

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Summary

The gradient separation conditions in two-dimensional liquid chromatography are usually used for improvement of the separation efficiency and for better utilization of the separation space. In the work, procedures for optimization of both, gradient two-dimensional liquid chromatography and liquid chromatography coupled to capillary electrophoresis were developed. The in-laboratory build software for processing of two-dimensional data was extended with the optimization protocols.

1 Introduction

The separation of complex samples of biological origin containing tens of even hundreds of analytes may be difficult or nearly impossible using a single separation method. The increased separation power is achieved using two-dimensional separations, which provide enhanced peak capacity with respect to the unidimensional methods. The proper optimization of such methods requires adjusting separation conditions in both dimensions, as well as conditions for efficient transfer of the fractions, with respect to the compatibility of both separation methods used. Under gradient separation conditions, orthogonality and peak capacity of the system can be significantly improved [1]. Due to the peak compression effects, the liquid chromatographic conditions with fast gradients provide higher efficiency with respect to the isocratic separation mode [2]. The protocols for optimization of the two-dimensional separations employing liquid chromatography in both separation dimensions and liquid chromatography in combination with capillary electrophoresis were developed in this work.

2 Experimental

The experimental data used in the study were obtained using two-dimensional methods described elsewhere [3]. The developed optimization protocols were incorporated into the software for processing of two-dimensional data, Eval2D, developed in our laboratory and programmed using Python object-oriented language (version 3.3.5.) with Matplotlib plotting library (version 1.3.1.). The mass spectrometric data in combination with pressure-driven and electrodriven separations were acquired using AB SCIEX QTrap 4500 mass spectrometer (SCIEX, Framingham, MA, USA) operated in ESI mode.

3 Results and Discussion

The mathematical models suitable for description of retention data were developed for hydrophilic interaction liquid chromatographic separation of oligomeric series of analytes [4]. The numerical integration together with the iteration process was used for the calculation of gradient retention times [5]. The gradient data were combined together for two-dimensional LCxLC system; under LCxCE conditions, the electrophoretic separations were selected with respect to the highest peak capacity and highest peak-production rate. The effects of the composition of first dimension eluate were compensated using gradient conditions in the second dimension for both, liquid chromatography and capillary electrophoresis. Thus, the protocol for optimization of changing composition of background electrolyte for the analysis of consecutive fractions transferred from LC was developed. Finally, the gradient optimization procedures were incorporated into the software developed in our laboratory for processing of experimental data obtained by two-dimensional pressure-driven and electrodriven separations, coupled to either single-channel, or multichannel detection techniques (i.e. in combination with mass spectrometry).

4 Conclusions

Two-dimensional liquid chromatography and liquid chromatography in combination with capillary electrophoresis are viable methods for analysis of highly complex samples. Optimized gradient conditions used in both dimensions significantly improve the separation performance of the method, increase compatibility of phase systems and utilization of separation space.

Acknowledgement

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P10 EVALUATION OF STATIONARY PHASES FOR HPLC BASED ON NANOFIBERS

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Summary

Due to their properties nanofibers have the potential to be a good sorbent in separation techniques. In this study, the performance parameters were compared for the set of chromatographic columns filled with polyacrylonitrile nanofibers processed in the different manner. This material has been used as non-treated and pulverized mat, as well stacked or rolled membrane. The advantages and challenges of the novel fillings were discussed.

1 Introduction

Nanomaterials are particles possessing at least one dimension in the nanometer range. In the case of nanofibers (NFs), the crucial dimension is the diameter that typically varies in the limit of tens to hundreds of nm. NFs are made from inorganic (oxides, metals, carbon), organic (synthetic polymers, biopolymers) materials or mixtures of them [1]. The leading technology of its production represents electrospinning based on

the combination of electrostatic and mechanical forces pulling the polymer solution from the reservoir to the grounded collector. It has been established as a simple, versatile, and scalable technique to produce nanofibers with tailor made properties [2].

The main advantages of NFs are unique structure and properties (physical, chemical, and biological), such as large surface area to volume ratio, small pore size, high porosity, good breathability, and stable structure. The above mentioned ratio along with the wide variety of chemical and morphological modifications predetermined it for the application in the field of analytical chemistry in sensors, separation membranes, and stationary phase for solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC). During the last decade SPE on nanofibrous phases has become an important and efficient purification technique, especially for small molecules in medicinal and environmental analysis. Frequently they are used as: electrospun nanofiber mats in tips, filter holders or solution [3]. The separations on HPLC beds consisted of NFs are reported very rarely, in spite of the fact that fibrous stationary phases with thicker fiber diameter provided promising separation of macromolecules [4].

2 Experimental

NF samples (polyacrylonitrile mat and membrane) were obtained from Pardam (Roudnice nad Labem, Czech Republic). Chromatographic columns were filled with the fibrous bed (about 0.7 g per column) and compressed using home-made tool. For the purposes of analysis, NF mat were treated with ultrasound homogenizer and grinded frozen in mortar. Degree of fiber disruption was monitored using scanning electron microscopy MIRA3 (Tescan, Czech Republic). NF membrane were either rolled on metal wire and pulled into the column, or divided into the circles and stacked up in column body.

HPLC experiments were performed on the prepared nanofibrous columns (100 × 4,6 mm) and commercial one (Eclipse XDB-C8, 5 μm, Agilent). Mobile phase consisted of acetonitrile and water, performance characteristics were obtained for acetone as void volume marker. Chromatographic analyses were carried out with 1100 series instrument coupled with diode-array detector (Agilent).

3 Results and Discussion

The chromatographic performance of nanofibrous stationary phase was studied on the model of polyacrylonitrile. Due to the presence of polar and non-polar sites, more separation modes should be used (reverse phase vs. hilic).

There were compared three different filling of the column with nanofiber mat: untreated and disintegrated manually or after using ultrasound device. The aim of the treatment was homogenization of the bed leading to tight placement of fiber fragments. Back pressure were analyzed by using mobile phases of different compositions with lowest value obtained for intact fibers (comparable to no-column or empty column arrangement; Fig. 1) and high-organic solvent. Specific permeability K^0 of this column reached $9.64 \times 10^{-8} \text{ mm}^2$ for 50% acetonitrile, while other ones were 5 to 8 times lower. Subjected to higher mobile phase velocities ($>0.5 \text{ mL/min}$), as well percentage of water

(>50 %), the fibrous bed intensively settled, especially for untreated fibers. The emerging gaps on the front of columns lead to peak broadening and shoulders.

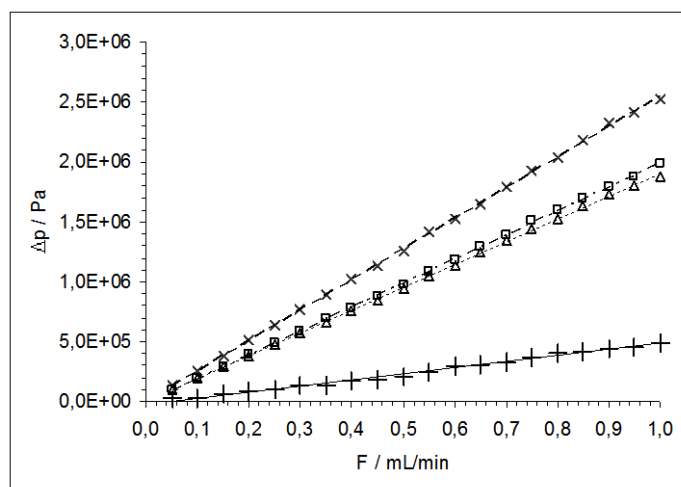


Fig. 1. Effect of mobile phase velocity on the back pressure of NF columns (+ non-treated, × grinded, and Δ sonicated fibers) vs. C8 column (□).

Number of theoretical plates (n) and symmetry of the peak were compared for acetone. Apparently higher n -value provided NFs treated with ultrasound, although it was barely reached of 5 % of C8 (particulate bed) column. The higher homogeneity of ultrasound treated NFs was supported by the pictures from SEM showing shorter fragments in narrower distribution than in the case of grinded ones which consisted of fragments differ in length, with some pieces flattened.

To stabilize the position of NFs in the column, more rigid arrangement of fibers, membrane, has been used. Moreover, membrane plates were oriented perpendicular to the column axis (parallel disks) or rolled around the axis (like a cigar). The results for the former one were similar to non-treated mat including back pressure and settling of the fibers. The latter arrangement provided relatively sharp peaks (similar to ultrasound treated NFs) with longer retention times. An explanation of the observation should be based on more organized and compact bed of the corresponding column.

4 Conclusions

The results showed the best used alignment of NFs was random mat with sequential loading under increased pressure. In this case, the settling of the stationary phase was reduced. On the other hand, the grinded NFs provided the highest efficiency, but the worst resistance to back pressure. Another perspective, how to stabilize the bed seems to be homogenous and tight rolled fibrous membrane fixed in column.

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P11 DETERMINATION OF LEVOGLUCOSAN IN URBAN AEROSOL

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Summary

The main aim of this work was a development of the analytical method for levoglucosan determination including direct collection of aerosol into water phase, preconcentration, and determination by LC-MS.

1 Introduction

Monosaccharide anhydrides are frequent water soluble organic compounds (WSOC) in fine fraction of the atmospheric aerosol. The most abundant compound among them is levoglucosan, a product of cellulose combustion. The mannosan and galactosan, the isomers of levoglucosan formed during combustion of hemicellulose, are present in aerosol in minor amount.

2 Experimental

The collection of levoglucosan was performed by an improved version of the Aerosol Counter-flow Two Jets Unit (ACTJU) aerosol collector [1]. A combination of the original version of ACTJU collector with water-based condensation growth tube (GT) located upstream of the ACTJU collector enabled quantitative sampling of aerosol particles down to a few nm in diameter [2]. Before LC-MS analysis, an optimization of separation and detection by LC-MS was carried out. The separation of levoglucosan, mannosan, and galactosan was performed with a polymeric amino column Prevail Carbohydrate ES (Grace, USA). To enhance ionization efficiency, and therefore to reduce the detection limits of target compounds, post-column additions of aqueous solutions with CH₃COOH, CH₃COONH₄, NH₄Cl, LiCl, and NH₃ in various concentrations were compared.

3 Results and Discussion

Commercial and home-made SPE columns were tested for the preconcentration of monosaccharide anhydrides. Besides, the preconcentration of the levoglucosan by a vacuum evaporator was optimized as an alternative approach. The highest increase in response ($[M+Li]^+$ ions) was induced by the addition of 1 mM LiCl (Fig. 1.). The values of detection limits of the method with LiCl postcolumn addition ranged from 0.3 $\mu\text{g/mL}$ (levoglucosan, mannosan) to 30 $\mu\text{g/mL}$ (galactosan).

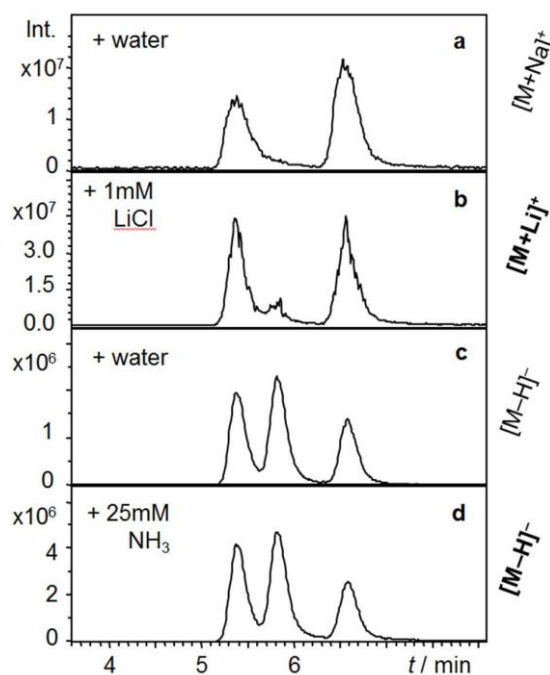


Fig. 1. Extracted ion chromatograms of ions corresponding to monosaccharide anhydrides after post-column addition in the positive (a, b) and negative (c, d) ion modes.

4 Conclusions

The analytical method for the determination of levoglucosan, mannosan, and galactosan using LC-MS was optimized. An improved design of aerosol collector was proposed for the quantitative collection of aerosol into the water. The method of levoglucosan preconcentration by means of vacuum evaporator was developed with the collection efficiency of 98.4 – 102.0 %.

Acknowledgement

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**P12 MULTIPLE HYPERNATION OF LC WITH ESI MS, MALDI
MS AND SALD ICP MS – THE DETECTION PLATFORM FOR
METALLOPROTEOMIC STUDIES**

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Summary

The combination of the molecular and elemental detectors for a single separation by utilizing on-line and off-line approach is presented. This arrangement is demonstrated on analysis of a metallothionein mixture.

1 Introduction

We present a new methodology for coupling conventional on-line LC-ESI MS with two off-line detection techniques: matrix-assisted laser desorption/ionization (MALDI) and substrate-assisted laser desorption inductively coupled plasma (SALD ICP) mass spectrometry (MS) analyses. The off-line approach is facilitated using a commercial MALDI target made of conductive plastics, which is suitable for the both MALDI MS and SALD ICP MS. Due to this arrangement, two types of analysis, molecular and elemental determination of metallothionein 1 (MT-1) were performed.

2 Experimental

LC separations were performed using a Vydac C8 (150 x 4.6 mm x 5 µm) column. For the separation of MT-1 isoforms (MT-1a, MT-2d, MT-2e), the effluent coming out of the HPLC column and passing via a UV detector was split with a MicroTee. One part of the flow was introduced on-line to ESI oTOF MS detector, the second part of the effluent was deposited on MALDI plastic targets (Prespotted AnchorChip⁹⁶) as 1.6-µL fractions using a tapered fused silica capillary (I.D. 30 µm). After drying at room temperature, dry fractions were overlaid with 0.5 µL of saturated CHCA solution in 20% ACN and 1% TFA (v/v) with In as an internal standard. Off-line MALDI TOF and SALD ICP quadrupole MS detection were performed from the same target.

3 Results and Discussion

Simultaneous detection of the MT-1 isoforms (complexes with metals), apoforms (forms without metals) and metal quantification were recorded using off-line and on-

line approach. The MT-1 complexes were separated at the neutral pH (7.0) and detected with ESI MS to obtain information about metal complexes and determine their metal-complex stoichiometry. Spotted fractions overlaid with acidified MALDI matrix were measured with MALDI MS for apoforn determination. Subsequently, the total metal content was quantified by SALD ICP MS from the same deposited fractions. On-line ESI MS provided information about the MT-1 complexes and their metal stoichiometry. MALDI MS offered additional information about protein apoforns, thus confirming ESI MS identification. SALD ICP MS supplied quantitative data on metals in the MT-1 complexes.

4 Conclusions

A new combination of on-line and off-line MS detection techniques, ESI MS, MALDI MS and SALD ICP MS, was demonstrated in a single separation run. A commercial MALDI plastic target was found to be applicable for the both MALDI and SALD ICP MS analysis. Using this approach both molecular and elemental determination of measured sample was obtained. In addition, two complementary soft ionization techniques, ESI and MALDI MS were compared.

Acknowledgement

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P13 CHARACTERIZATION OF FRET SENSOR

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Summary

In this study, we present characterization of sensor based on Förster resonance energy transfer (FRET). The sensor is composed of ssDNA chain attached to a laboratory synthesized quantum dot (QD). A complementary chain of a sample is labeled by a luminescent dye. When the dsDNA hybrid is formed, the energy from the QD (donor) is transferred to the dye (acceptor) and FRET is observed as a decrease of QD

luminescence emission intensity and an increase of dye luminescence emission intensity.

1 Introduction

One of the most important characteristics of the FRET sensor is FRET efficiency E_F representing a yield of energy transition. It can be calculated according to Eq. 1 from luminescence emission intensity of donor at the presence (F_{DA}) and at the absence (F_D) of acceptor [1].

$$E_F = 1 - \frac{F_{DA}}{F_D} \quad (\text{Eq. 1})$$

Another characteristic of the donor-acceptor pair is Förster distance R_0 . According the definition, the Förster distance and the real donor-acceptor distance are equal when FRET efficiency is 50 %. R_0 depends on refractive index of a surrounding medium N , orientation factor κ , the donor quantum yield Φ_D and spectral overlap integral J (Eq. 2).

$$R_0^6 = 8.79 \times 10^{-5} (N^{-4} \kappa^2 \Phi_D J) \quad (\text{Eq. 2})$$

Equation 3 shows dependence of E_F to Förster distance R_0 , real distance between donor and acceptor R and n represents number of acceptors bounded to one donor [2].

$$E_F = \frac{nR_0^6}{nR_0^6 + R^6} \quad (\text{Eq. 3})$$

Limit of detection (LOD) describes analytical quality of FRET sensor and is calculated from luminescence intensity of acceptor, which is three times higher than the standard deviation of the noise.

2 Experimental

We synthesized CdTe QDs by a modified two-step reaction [3]. Formed nanocrystals of QDs were coated by 3-mercaptopropionic acid (MPA) to make them negatively charged and water dispersed. Laboratory made QDs were attached to ssDNA *via* metal-thiol dative bonding. The QD-ssDNA conjugate was exposed to a model complementary ssDNA labelled with luminescent dye 6-carboxyrhodamine (ROX), which was purchased from Sigma-Aldrich, Germany. The hybridization was performed using a standard annealing protocol. Capillary electrophoresis with laser induced fluorescence detection (CE-LIF) and luminescence spectroscopy were used for analyses of each reaction step and FRET experiments as well.

3 Results and Discussion

To characterize developed FRET sensor, the emission intensity of donor (QD-ssDNA conjugate) at presence of acceptor (ROX-ssDNA) at various concentration levels were

evaluated. The increasing amount of ROX-ssDNA leads to the FRET efficiency increase until the value of saturation is reached.

The E_F value was calculated according to Eq. 1 and determined to 30 % for four molecules of ROX-ssDNA hybridized QD-ssDNA conjugate. Calculated Förster distance (Eq. 2) is 3.52 nm. For the system, where one donor is bounded with four acceptors, R distance between the donor and one acceptor is 5.11 nm.

4 Conclusions

In this work, characterization of developed FRET-sensor was presented. Four main sensor prosperities were calculated from luminescent measurements. For the system, where four acceptors are bound to one donor, the FRET efficiency was determined to 30%. Förster and real distance was calculated to 3.52 nm and 5.11 nm, respectively. Limit of detection was determined to 6 nM before PCR hybridization.

Acknowledgement

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P14 THE EFFECT OF PREANALYTICAL CONDITIONS ON HUMAN SERUM N-GLYCOME

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Summary

One of the most important protein post-translational modification is glycosylation. Methods often used for the analysis of released glycans include MALDI-TOF-MS and CE-LIF. Analytical methods usually undergo a very thorough optimisation process, however, the influence of the preanalytical conditions is often forgotten. In this work,

the effect of preanalytical conditions on the composition of the N-glycome was tested and conditions that can cause unreliable results were identified.

1 Introduction

Glycans take part in many cellular and biological processes such as immune reactions, cellular interactions and signaling.¹ Glycans isolated from human serum can be used as markers for cancer and inflammatory diseases.^{2,3} However, it is likely that activity of enzymes, such as exoglycosidases, influences the glycome. Therefore, it is important to investigate how the composition of the serum or plasma glycome is altered upon different collection and preanalytical processes e.g. type of collection, prolonged time before centrifugation, temperature before centrifugation, additives used in collection tubes.

2 Experimental

Blood from 10 healthy female donors (20-30 years) was collected in various collection tubes and further processed, resulting in 16 different conditions per person. Glycoproteins from serum/plasma were denatured, reduced and alkylated. N-Glycans were then released using PNGase F. N-Glycans were subsequently isolated by C18 reversed-phase microcolumns and purified using graphitized carbon cartridges. Half of the N-glycan pool was permethylated and analysed by MALDI-TOF-MS in the positive ionization reflectron mode, the rest was desialylated, labelled with APTS and measured with CE-LIF. The Mann-Whitney U-test was used to compare the different preanalytical conditions to the reference samples. P-values lower than 0.05 were considered statistically significant.

3 Results and Discussion

Samples were stable when collected by both vacuum and free flow as judged by MALDI-TOF-MS. Various storage conditions before and after centrifugation, heparin as additive, and 2-month storage at -20°C and -80°C also didn't influence the stability of N-glycome. Additionally, both serum and plasma samples produced the same results. Samples, which were stored for 2 months, differed from the reference samples in four glycan structures, including three high-mannose glycans, whose relative intensities were increased. Significant differences in relative intensities of five structures were observed for samples collected in EDTA collection tubes. The haemolysed samples showed significant differences in almost a quarter of all recorded glycans. These changes include an increase in relative intensities of high-mannose glycans and decrease of sialylated oligosaccharides.

4 Conclusions

It appears that the analytical protocol ensures repeatable results regardless of the preanalytical conditions. Small delays in centrifugation do not influence the results, but, to ensure better results, a storage at 4°C is recommended, when immediate centrifugation is not possible. Still, samples collected in EDTA and samples, which are visibly haemolysed should not be analysed, since these provide unreliable results.

Acknowledgement

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P15 MOLECULAR GLYCOPATHOLOGY

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1 Introduction

Formalin fixation combined with paraffin embedment is the most prevalent method to preserve tissues from degradation. This type of retained tissue is routinely prepared for pathological investigations, thus formalin fixed paraffin embedded (FFPE) tissue samples became the almost exclusively used for long-term storage in histopathological laboratories and hospitals. FFPE specimens can serve as a valuable alternative for fresh frozen biopsy samples, which should be stored at -80°C. The objective of our study was to perform global N-glycosylation profiling of FFPE mouse tissues using high performance capillary electrophoresis analysis with laser induced fluorescence and MS detection.

2 Experimental

SCID male mice were used for the analyses. Animal-model protocols were carried out in accordance with the Guidelines for Animal Experiments and were approved by the Institutional Ethics Committee at the National Institute of Oncology, Budapest, Hungary (permission number: 22.1/722/3/2010). The tissues were treated with formalin and embedded in paraffin. During the analysis process FFPE tissue samples were deparaffinized, solubilized with radioimmunoprecipitation assay (RIPA) buffer, digested with PNGase F enzyme, labeled with 8-aminopyrene-1,3,6-trisulfonic acid (APTS) fluorescent dye and measured by capillary electrophoresis laser induced fluorescent (CE-LIF) detection [1] and also with mass spectrometry (CESI-MS).

3 Results and Discussion

Mouse spleen, brain, heart, lung, kidney, liver and intestine were fixed with formalin, embedded in paraffin then analyzed with CE-LIF (Fig. 1.). APTS labeled maltose was used as internal standard for the alignment of the electropherograms.

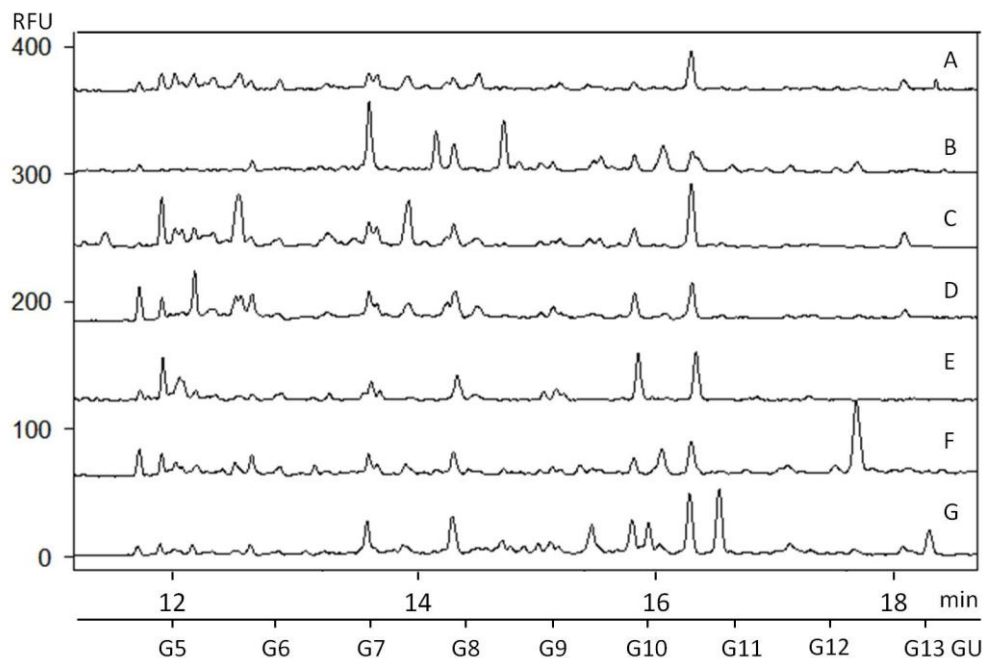


Fig. 1. Total N-glycan profile from FFPE mouse organs A) lung B) brain C) heart D) spleen E) liver F) kidney G) intestine.

The total N-glycan pools were sequenced with exoglycosidase enzymes. The GU values and the sequence information were compared to an in-house generated N-glycan database (GUcal). MS detection of selected CESI separated samples was also attempted.

4 Conclusions

So far the huge FFPE sample collections located in hospitals all around the world, were occasionally used for genomic and transcriptomic analyses in addition to routine pathological studies. Now the growing need to find biomarkers for various diseases requires using FFPE tissue specimens in other omics fields as these samples represent a rich source for retrospective and prospective biomedical and biopharmaceutical studies.

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P16 CAPILLARY ELECTROPHORESIS AS A TOOL FOR DIAGNOSIS OF METHANOL AND ETHYLENE GLYCOL POISONING FROM BLOOD SAMPLES

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Summary

A simple and fast capillary electrophoretic method with contactless conductivity detection (C4D) for analysis of oxalate, formate and glycolate in blood plasma samples is presented. Target analytes of metabolism of methanol and ethylene glycol are separated from each other and from other analytes present in blood plasma samples in less than 1.6 min. The separation electrolyte is composed of 20 mM 2-(N-morpholino)ethanesulfonic acid (MES), 20 mM L-histidine (HIS), 2 mM 18-Crown-6 and 30 μ M cetyltrimethylammonium bromide (CTAB), pH 6. For analysis of blood plasma samples in point-of-care analysis, a blood plasma separation device, which can operate without use of electricity was developed.

1 Introduction

Intoxication by methanol and ethylene glycol belongs to the most common intoxication that requires prompt diagnosis and initiation of treatment plant to prevent morbidity or mortality. The parent alcohols are much less toxic than their metabolites, notably formic, glycolic and oxalic acids that are responsible for various symptoms including loss of vision, CNS depression, renal failure etc. [1].

2 Experimental

2.1 Electrophoretic system

A purpose-built CE instrument was composed of power supply unit (DX250, EMCO high voltages, Sutter Creek, CA, USA). For all measurements was use voltage of -15 kV. All separations were conducted in fused-silica separation capillaries (50 μ m ID, 375 μ m OD, 40 cm total length, Microquartz GmbH, Munich, Germany). For detection of analytes, a custom made C4D detector, ADMET (Ver. 5.06, ADMET, Prague, Czech Republic and AD converter (ORCA 2800, ECOM s.r.o., Prague, Czech Republic) were used. The optimized background electrolyte BGE consisted of 20 mM MES, 20 mM HIS) 2 mM 18-Crown-6 and 30 μ M CTAB, pH 6.

2.2 Blood plasma separation device

For preparation of blood plasma samples, a device which can operate without use of electricity was developed. A sufficient volume of blood plasma samples needed for CE analysis was obtained. Apart from whole blood taken by venipuncture, a whole blood from fingerprick is also useful for preparation of blood plasma since the required volume is as low as 5 μl . Pending patent application.

3 Results and Discussion

3.1 Optimization of the separation electrolyte system

An optimization of the separation electrolyte system was conducted to separate the peaks of oxalate, formate and glycolate from the other peaks of the compounds that can be present in the various body fluid samples. With the help of PeakMaster 5.3 freeware, several background electrolytes were simulated to separate the analytes of interest. Best results, confirmed also experimentally, were obtained with electrolyte mentioned in section 2.1 *Electrophoretic system*.

3.2 Analytical parameters of method

The analytical parameters of the developed CE method for oxalate, formate and glycolate were investigated. The calibration curves were strictly linear in the range of 0–100 μM . They are shown in Figure 3. The R^2 ranged from 0.9975 to 0.9986. LODs ranged from 0.40 μM to 1.25 μM , LOQs from 1.33 μM to 4.17 μM , RSDs of time from 0.3 % to 0.7 % and RSDs of peak areas from 2.9 % to 3.1 %.

3.3 Analysis of real samples

The blood plasma samples, prepared from whole blood by the developed blood plasma separation device, were measured by CE-C4D. The samples were diluted with DI water before analysis. Electropherograms of standard mixture of anions in water, blood plasma without and with addition of standards of oxalate, formate and glycolate (each 10 mM in blood) are shown on Figure 1.

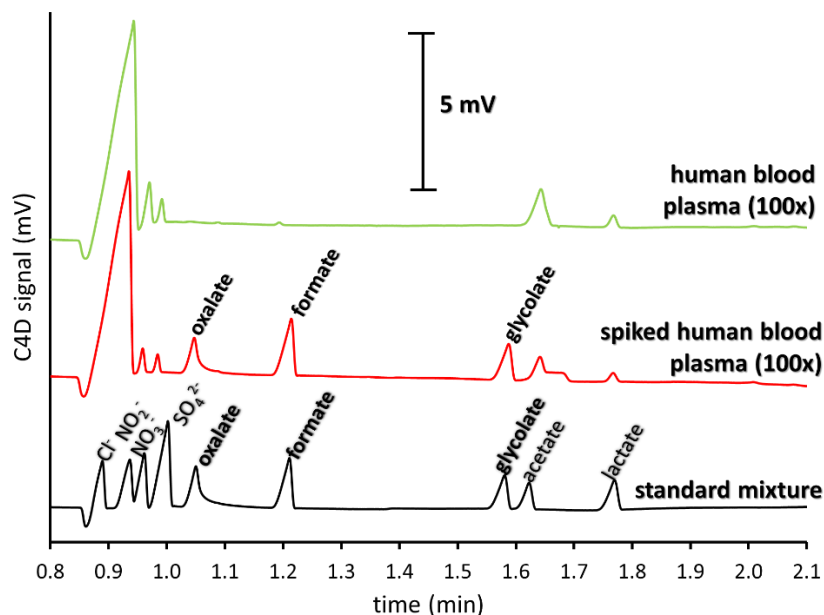


Fig. 1. CE-C4D separation of model mixture of 9 anions - each 50 μM (black); blood plasma spiked with oxalate, formate and glycolate (red) and blood plasma (green).

4 Conclusions

A simple and rapid capillary electrophoretic (CE) method, using contactless conductivity detection (C4D), for simultaneous analysis of methanol (formate) and ethylene glycol (oxalate and glycolate) metabolites from a drop of blood was developed. Furthermore, we present here a new, simple device that can be used to separate the blood plasma from as little as 5 μL of whole blood in less than a minute, using no electric power. This may be important especially in the point-of-care analysis.

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P17 A POTENTIAL OF USAGE NON-HAZARDOUS OPTICAL PROBES FOR CHARACTERIZATION OF VARIOUS OPTICAL DETECTORS

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Summary

To get an information about effective pathlength, % of stray light, sensitivity and linearity of optical detectors is not simple for every cases. Sometimes is the information different from manufacturer, or is hard to estimate it. The experimental methods include determination all of written parameters done by one experiments use hazardous compounds like chromate and is limited by usage specific wavelength. This work presents a potential of next optical probes to replace hazardous compounds and to test them in various types of optical detectors.

1 Introduction

A characterization of optical detectors gives an important contribution for evaluation of analytical methods. Especially information about linearity of detectors and effective pathlength are the most important parameters in optical detections. Those two parameters should be experimentally checked because are dependent on analytical set-up and can be different from manufacturer notes. Also, due development of new types of optical detectors, or their re-assembling, is necessary to determinate a sensitivity, a linearity and an effective pathlength values. Sometimes is not trivial to calculate them simple just from geometry because next additional effects, like reflectance, dispersion or absorption of light signal can be present. Manufacturer sometimes do not provide those information and it is hard to estimate those value, because the detectors in instrumental set-up are cover “black-boxes”.

For the determination of an effective pathlength and linearity of detector is possible to use the simple method which Macka et.al. published in 1996 for LED detector used on-capillary detector in capillary electrophoresis [1]. The advantage of this method is to provide several information by one measurement. From one plot is possible to determinate of the sensitivity, linearity of detector, % of stray light and effective pathlength [2]. The broader area of usage of this method on various instruments with different type of detectors has been used on 2003 by Johns et.al [3].

The simplicity to obtain of quick and important results is undeniable, but there is a couple of disadvantages over published works. The different type of light (used wavelength), require different optical probes with suitable chromophores. Optical probes used in published work are classified between hazardous chemical substances, e.g. chromate, which may complicate scientific work with them especially in the countries with strong health policy.

This work present a selection and experimental test of compounds which can be used for characterization of optical detectors and are not-hazardous, are commercially available and require conditions for comfort work in lab, like solubility in water, stable and have enough absorption of the light.

2 Experimental

Compounds potentially usable like optical probes has been selected according requirements – non-hazardous impact, solubility in water, molar absorptivity for specific wavelength, commercial availability and low price. For comparison of different selected compounds was used fused silica uncoated capillary (100 μm i.d.) with the stripped polyimide window incorporated into LED detector described in [1]. Several optical probes – Chromate(VI) dissolved in 50 mM NaOH, Brilliant Blue and Tartrazine solved in water were tested. Some of those compounds were tested on various types of detectors include nanoflow Z-cell (Knauer, Germany) and multichannel fused silica capillary – Photonic crystal fibers (PCFs) (NKT Photonics A/S, Denmark).

3 Results and Discussion

The table of selected compounds potentially used for replace of the hazardous chemical is shown. Available characteristics of compounds are summarized. There was focus on characteristic of compounds which should replace the hazardous compounds without loss of their efficiency. According this table were selected the most suitable compounds, which were check experimentally for determination of absorption coefficients and then were tested like optical probes for determination of detector characteristic. The most interesting optical probes shows food dyes. Especially for deep-UV area and also for blue light (470 nm) is suitable Tartrazine. It is perfectly soluble in water and gives a strong absorption around 255 and 470 nm. A comparison with chromate, which has been used in previous work is shown. Those optical probes has been demonstrated on on-capillary LED detector where the effective pathlength was close to noted internal diameter (100 μm) and % of stray light was less than 1 %. The test of optical probes on multichannel fused silica capillary and on nanoflow Z-cell like non-trivial optical cell is demonstrated.

4 Conclusions

A couple of potentially usable optical probes for replacing of a hazardous chemical compounds (e.g. chromate) is presented. The selected optical probes were tested in various optical LED detectors. Selected food dyes, like a new optical probes, were selected for replacement of hazardous compounds (Chromate).

Acknowledgement

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P19 COMPARATIVE EVALUATION OF PROTEIN COMPOSITION IN HUMAN BREAST CANCER CELLS USING MASS SPECTROMETRY

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Summary

Bottom-up proteomic approach was used for detailed characterization of proteins from two human tumour cell lines representing major clinically different types of breast cancer. The aim was to show the differences between them on proteomic level. Here we present almost 100 unequivocally identified proteins out of which 60 were mutually differently expressed for MDA-MB-231 and MCF-7. Some well-known breast cancer markers like annexins A1, A2 and vimentin were found in the MDA-MB-231 cell line. On the other hand, MCF-7 cells were found to be positive for cytokeratins and keratins and thus we were able to distinguish both cell lines sufficiently.

1 Introduction

MCF-7 and MDA-MB-231 are the most commonly used breast cancer cell lines in the present cancer research. Both lines originated from pleural effusions of metastatic mammary carcinoma patients [1]. MCF-7 line is an ideal model for studies of hormone response because it has high hormone sensitivity through expression of estrogen receptor [2]. The MDA-MB-231 line is used as a model for triple negative breast cancers [1].

Given that proteins are important indicators of physiological or pathological states and may contribute to the early diagnosis of diseases [3], the knowledge of

structure, function and amount of specific proteins is crucial and developing of effective diagnostic approaches to human cancer with perspective in malignances diagnostics and therapy is still needed.

Various proteomic tools allow functional investigation of the proteome [4]. Furthermore, mass spectrometry based proteomics has potential to give robust identification of unknown proteins as well as to provide a powerful view of the proteome including the qualitative and quantitative changes of proteins [5].

2 Experimental

2.1 Cell culture

The cancer cell cultures were purchased from the HPACC (Salisbury, Great Britain). Cells were grown and passaged routinely as monolayer cultures. For experiments, the cells were used at passage 10–30. Cells were seeded in Petri dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, antibiotics and cultured in humidified atmosphere of 5% CO₂ and 95% air at 37°C. After incubation, the cells were washed with ice-cooled PBS. The cell lysis was made according to manual instruction from the RIPA buffer by Sigma. The cell lysates were stored at –70°C for further use.

2.2 1D SDS-PAGE

The samples were dialyzed against deionized water using Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific, Waltham, MA, USA) with 2 kDa cut-off and lyophilized. Purified samples were applied onto the 12% SDS gel visualized using Coomassie Brilliant Blue G-250 dye.

2.3 In-gel digestion

The protein spots were excised from the gel and in-gel digested with trypsin (digestion buffer: 50 mM NH₄HCO₃, 5 mM CaCl₂, 12.5 ng/μL of enzyme) overnight at 37°C. The resulting tryptic peptides were extracted from the gel by 0.1% trifluoroacetic acid (TFA) and acetonitrile (1:1, v/v). For mass spectrometric analyses, the extracts were purified by ZipTip C18 (Millipore).

2.4 Mass spectrometry and database searching

A solution of α -cyano-4-hydroxycinnamic acid (8 mg/ml in acetonitrile/0.1% TFA, 1:1, v/v) was used for both MS and MS/MS analysis of peptides. MALDI MS experiments in positive ion reflectron mode were performed on AB SCIEX TOF/TOF™ 5800 System (AB SCIEX, Framingham, MA, USA). Acquired mass spectra were processed using 4000 Series Explorer software and the data were submitted to the Mascot database searching. Protein identifications were assigned using the NCBI nr database with taxonomy restriction to Homo sapiens. Maximum tolerance for peptide masses as well as fragment error was set to 0.3 Da. Additional parameters used: enzyme trypsin; allowed missed cleavages: up to one, fixed modification: carbamidomethyl, no variable modification; peptide charge: +1; monoisotopic masses; instrument MALDI-TOF/TOF.

3 Results and Discussion

The combination of 1D gel electrophoresis with MS was applied to distinguish the differences in the protein profile of MCF-7 and MDA-MB-231 human breast cancer cells. We analyzed the cell lysates of these model human tumor cell lines representing two major clinically different types of breast cancer. Almost one hundred proteins were positively found, while 60 of them were expressed differentially between MDA-MB-231 and MCF-7 cell lines (as an example of several identified proteins see Table 1).

Table 1. Selection of identified proteins obtained by MS measurement (mark ✓: cell line consisting of the protein).

Cell line		Identified protein	NCBI nr
MCF-7	MDA-MB-231		
✓	✓	Calreticulin=calcium binding protein	gi 913148
✓	-	Glucose-6-phosphate dehydrogenase	gi 26224790
✓	-	UDP-glucose-6-phosphate dehydrogenase isoform 1	gi 4507813
✓	-	Peptidyl-prolyl-cis-trans isomerase FKBP4	gi 4503729
-	✓	Vimentin	gi 340219
-	✓	Calreticulin precursor	gi 4757900
✓	✓	Tubulin alpha-1B chain	gi 34740335
✓	✓	Tubulin beta-4B chain	gi 5174735
✓	✓	Tubulin beta-5 chain	gi 7106439
✓	✓	Cytokeratin 8	gi 181573
✓	✓	ATP synthase subunit beta, mitochondrial precursor	gi 32189394
✓	-	Beta-tubulin	gi 338695
✓	-	Alpha- tubulin	gi 37492
-	✓	Annexin A1	gi 4502101
-	✓	Annexin A2	gi 18645167
-	✓	Keratin 10, partial	gi 186629
✓	-	Cytokeratin 8	gi 181573
✓	-	Cytokeratin 18 (424 AA)	gi 30311

These identified proteins can be sorted into several categories based on their major biological functions. Actins, actinins and tubulins represent major proteins observed in both studied cell lines. The most numerous group of heat shock proteins is included also in both cell lines. Among the proteins reported in this work, some well-known breast cancer markers, e.g., annexin A1, annexin A2 or vimentin were detected in the MDA-MB-231 cell line. Especially vimentin, a specific protein for MDA-MB-231, has been known to participate in a number of crucial functions [6]. In contrast, only MCF-7 cells were found to be positive for some cytokeratins and keratins. This observation is consistent with results of Whelan [7] who described keratin 8 and 18 as other potential biomarkers in conjunction with keratin 19.

4 Conclusions

Here we applied simply comparative proteomics to discriminate the changes in the protein composition of MDA-MB-231 and MCF-7 human breast cancer cells. The significant differences between individual lines were mainly observed in cases of vimentin and annexins. Both mentioned proteins are supposed to play an important and crucial role during tumor cell growth and proliferating, especially they are related with the carcinogenesis process and metastasis formation in many tumors.

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P20 POTENTIAL OF NON-AQUEOUS CAPILLARY ELECTROPHORESIS- MASS SPECTROMETRY IN ANALYSIS OF OXYSTEROLS

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Summary

The potential of non-aqueous capillary electrophoresis (CE) coupled to mass spectrometry (MS) to analyze derivatized oxysterols is shown. The developed method was successfully applied for separation of derivatized oxysterols from excess derivatization reagent, resulted to simplified sample preparation (i.e. no need for SPE extraction before LC-MS analysis) and hence to reduction of total analysis time and sample handling. Separation of 25-hydroxycholesterol/27-hydroxycholesterol in model sample solution is shown.

1 Introduction

Oxidized metabolites of cholesterol, oxysterols (OHC), are important in numerous biological processes and pose multiple roles in the body. A variety of isomers exists, with different biological roles, e.g. 27-hydroxycholesterol (27-OHC) is a selective estrogen receptor modulator. Other isomers are for instance: 24S-OHC, 25-OHC, 7 β -OHC, 22R-OHC, etc.). 27-OHC can promote proliferation of estrogen receptor (ER) positive breast cancer by binding to ER and metastasis by binding to liver X receptor. Traditionally, separations of oxysterols are performed by gas chromatography (GC) or liquid chromatography (LC) [1,2] after derivatization with MS detection. Separation of oxysterols by aqueous CE is difficult mainly because of their low solubility in water and levelling effect of water. In this study we explore the potential of non-aqueous capillary electrophoresis coupled to mass spectrometry (NACE-MS) for better separation of the oxysterol isomers.

2 Experimental

All CE-MS experiments were performed on amaZon SL Ion Trap Mass Spectrometer (Bruker, USA) in positive ionization mode. For CE-MS connection, an electrospray tip (CE ESI Sprayer II, Agilent, USA) was used. Fused-silica capillaries (50/30 μ m id, 360 μ m od, 80 cm length, Polymicro Technologies, Phoenix, AZ, USA) were used for the separation. The high voltage power supply (EMCO DX250R, USA) was used to deliver separation voltage (16.6 kV). Standards and cell samples were injected hydrodynamically at 35 cm for 15 s. The optimized BGE composition used in this work was 20 mmol/l ammonium acetate, 0.5 % 1 mol/L acetic acid in MeOH/ACN 1:1.

Sheath flow liquid consisted of 50% MeOH (in water) or MeOH/ACN 1:1 at flow rate 4 $\mu\text{L}/\text{min}$ was used.

3 Results and Discussion

3.1 Derivatization of oxysterols

Non-derivatized oxysterols and CE are not compatible, due to non-ionic character of oxysterols at different pH values. In addition, due to their neutral nature, oxysterols are not easily ionized with ESI. Therefore, a derivatization reaction with charge-carrying reagent (e.g. using Girard T reagents, Figure 1) is necessary in order to provide for the positive charge.

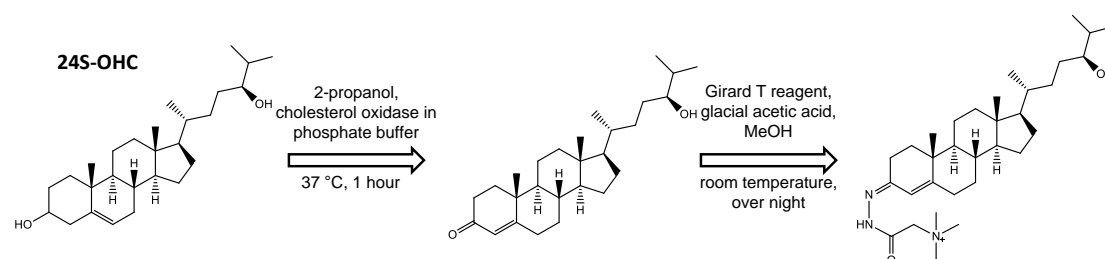


Fig. 1. The structure of 24S-hydroxycholesterol (24S-OHC). Charge-tagging of 24S-OHC with Girard T reagent.

3.2 Separation of reaction product from Girard T reagent

After the derivatization reaction of oxysterols with Girard reagent, the reagent is still present in high amounts in the solution. This is often a problem (especially when the large volume injections are performed in LC it causes high ion suppression) and off-line or on-line sample cleanup (solid phase extraction) is needed before LC analysis. This avoids the loss of sensitivity and resolution. This is not an issue in CE, where Girard T reagent and oxysterols have different mobilities resulting in different migration times and therefore no sample cleanup is needed before injection and analysis (Figure 2).

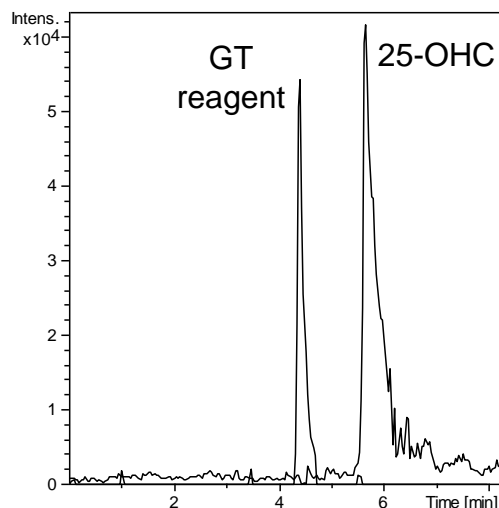


Fig. 2. MS signal of 10 $\mu\text{mol/L}$ 25-OHC and residue of Girard T reagent. Capillary: 70 cm long, 50 μm ID. Sheath liquid 50% MeOH in water. Scans were performed as SIM scan in MS mode. The monitored MS masses were 514.44 for 25-OHC and 132.19 for Girard T reagent.

3.3 Separation of 25-OHC and 27-OHC

Separation of oxysterols by CE is not trivial, mainly because oxysterols differ from each other only by different position of the hydroxyl group within molecule. To this day, by the developed method, we were able to separate only 25-OHC from other oxysterols (24S-OHC, 27-OHC, 22R-OHC and 7 β -OHC). Samples containing 1 $\mu\text{mol/L}$ 25-OHC and 1 $\mu\text{mol/L}$ 27-OHC were analyzed separately, and signals were compared (Figure 3). Similar results were obtained when 25-OHC and 27-OHC were analyzed as one mixture sample (data not shown).

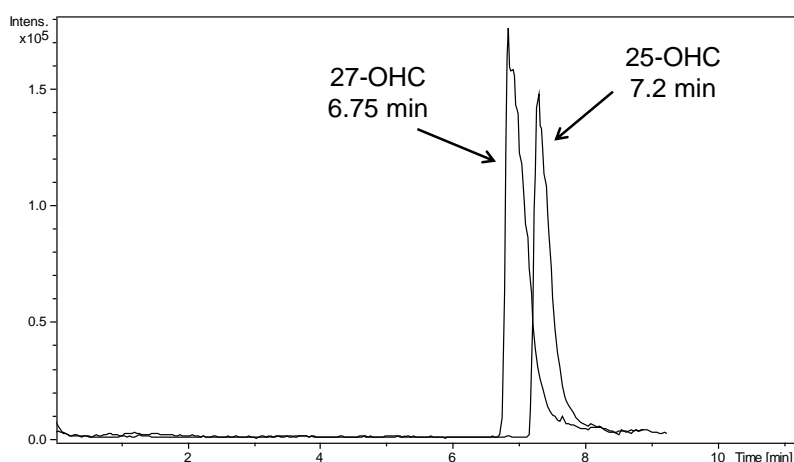


Fig. 3. MS/MS signal of 1 $\mu\text{mol/L}$ 25-OHC and 27-OHC. Capillary: 80 cm long, 30 μm ID. Sheath liquid 50% MeOH in water. MS detection was performed in MS/MS mode. The monitored MS/MS transitions were based on the fragmentation of the Girard T group and were 514.44 \rightarrow 455.36.

4 Conclusions

Our pilot study together with presented data shows a good potential of NACE-ESI-MS in analysis of oxysterols. One of the advantages of this technique is no need for complicated sample clean-up preparation, resulting in simplified sample handling and reduction of total time necessary for analysis. However, in order to improve the resolution and separation of other oxysterols further investigation is needed. Next step will consist of the separation and detection of oxysterols in real samples – in exosomes derived from cancer cells and cancer cells.

Acknowledgement

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P21 LIQUID PHASE SEPARATION METHODS FOR N-GLYCAN ANALYSIS OF BIOPHARMACEUTICALS AND HUMAN BIOLOGICAL SAMPLES

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Summary

Carbohydrate analysis of glycoproteins from human biological samples and biotherapeutics are important from diagnostic and therapeutic points of view. We summarized the latest liquid chromatographic and capillary electrophoresis techniques used in N-glycan analysis. Miniaturization of these methods is also important to develop high throughput separation methods and decrease analysis time.

1 Introduction

Recombinant protein-based drugs (e.g., monoclonal antibodies) are the fastest growing sector in the pharmaceutical industry. The biosynthesis of covalently attached

oligosaccharides (glycans) is often the most difficult to control during the production of recombinant glycoproteins, so to ensure quality, safety, and efficacy their glycosylation pattern should be carefully monitored during every stage of the manufacturing process. Glycans can also be used in clinical diagnostic as potential biomarkers. We summarized the latest liquid phase separation techniques used in N-glycan analysis [1].

2 Discussion

2.1 Liquid chromatography

Plenty of liquid chromatographic separation techniques have been developed during the past decades for the analysis of N-glycans. Normal phase (or hydrophilic interaction), reverse phase, adsorption (porous graphitized carbon), weak anion exchange chromatography and lectin affinity chromatography are the most common ones. Recently, developments in liquid chromatography moved toward miniaturized techniques, namely nano-LC or microchip-LC. The main advantages of nanoscale LC methods are high separation efficiency and resolution, rapid analysis time and minimal reagent and sample consumption.

2.2 Capillary electrophoresis

Capillary electrophoresis has been widely used for rapid separation of fluorophore-labeled glycans either in free solution or in gel-filled capillaries for decades. Miniaturized form of capillary electrophoresis, the microchip electrophoresis (ME) has the potential to develop high speed, high throughput, cost-effective and portable analytical systems. Integration, along with low sample/reagent consumption are some of the main advantages of ME. Detection of carbohydrates in biological samples by ME can be problematic due to their low concentrations and matrix interferences, thus sample pre-concentration steps are usually necessary prior to analysis.

3 Conclusions

The glycosylation pattern of glycoprotein based drugs affects quality, safety, and efficacy, therefore it should be continuously monitored at all stages of the development process. On the other hand, glycans are promising biomarkers, because malignant transformation and cancer progression cause aberrant glycosylation patterns. We can reduce analysis time and decreases reagent/solvent consumption by miniaturizing LC and capillary electrophoresis to microfabricated devices. Miniaturized separation methods can be easily integrated in the development process of biopharmaceuticals and also serve as point of care devices.

Acknowledgement

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P22 INVESTIGATION OF BIOACTIVE PROTEINS IN PRESSURIZED WATER EXTRACT OF *SAMBUCUS NIGRA* L. BRANCHES

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Summary

Investigation of high-value substances in plant materials has been of increasing interest in last years. In this study, PHWE, SDS-PAGE and MALDI-TOF techniques were employed for investigation of bioactive proteins in *Sambucus nigra* L. branches.

1 Introduction

Sambucus nigra L. (commonly known as black elder) is a wide-branched shrubby tree growing up to 10 m. It grows in hedgerows, woods, coppices, and waste places throughout the Europe, Asia, North Africa, and the United States. All parts of the plant have been used for generations in folk medicine because of their essential positive effects on the human diet and health, which could be mainly ascribed to the presence of several taste and health related compounds such as sugars, amino acids, organic acids, polyphenols and bioactive proteins [1, 2].

The objective of this study was the extraction of bioactive proteins by pressurized hot water (PHWE) from *S. nigra* L. branches, their separation by SDS-PAGE and identification by MALDI-TOF/TOF MS.

2 Experimental

2.1 Plant material

The braches of *S. nigra* L. growing in the Czech Republic were collected during autumn 2013, dried, ground to 6 mm size and stored in brown glass vials at laboratory temperature.

2.2 Extraction of *Sambucus nigra* L. branches

The pressurized hot water extraction (PHWE) was performed in laboratory-made extraction apparatus described before [3] at static arrangements. A portion (4 g) of ground branches was put into 22 mL stainless steel extraction cell and extracted at a pressure of 15 MPa and a temperature of 60 °C for 5 min followed by purging with nitrogen for 90 s. The resultant extract was left to cool down at room temperature and further processed as described below.

2.3 SDS-PAGE

Four hundred microliters of each water extract was mixed with 1.6 ml of ice-cold acetone. Samples were incubated overnight at -20 °C and then centrifuged at 14,000 g for 10 min. The supernatant was carefully removed and the pellet was dissolved in 50 µL of the sample buffer. After brief boiling, 12 µl of each sample was applied onto the polyacrylamide gel (stacking gel 4% T, separating gel 12% T) and the gel electrophoresis was performed on Mini-PROTEAN 3 Cell (Bio-Rad, Hercules, CA, USA). Constant voltage of 160 V was applied to run the gel. Proteins were visualized with CBB G-250.

2.4 MALDI-TOF/TOF analysis

A solution of α -cyano-4-hydroxycinnamic acid (8 mg/ml in acetonitrile/0.1% trifluoroacetic acid, 1:1, v/v) for dried-droplet preparation was used for both MS and MS/MS analysis of peptides. MALDI MS experiments in positive ion reflectron mode were performed on AB Sciex TOF/TOF 5800 System. MS/MS experiments were carried out by means of low-energy collision-induced dissociation (collision energy set at 1 keV) where ambient air was used as a collision gas. Acquired mass spectra were processed using 4000 Series Explorer software (AB Sciex, version 4.1) and the data were submitted to the Mascot database searching. NCBIInr database was used for the search with taxonomy restriction to “other green plants”.

3 Results and Discussion

The proteins were extracted from the ground branches by PHWE at 60 °C, which was found an optimal temperature for extraction of proteins of interest. Higher temperature leads to protein degradation while lower temperature is not enough to extract all the proteins from the material. After extraction, the proteins were precipitated with acetone and separated by SDS-PAGE. The protein pattern is shown in Fig. 1.

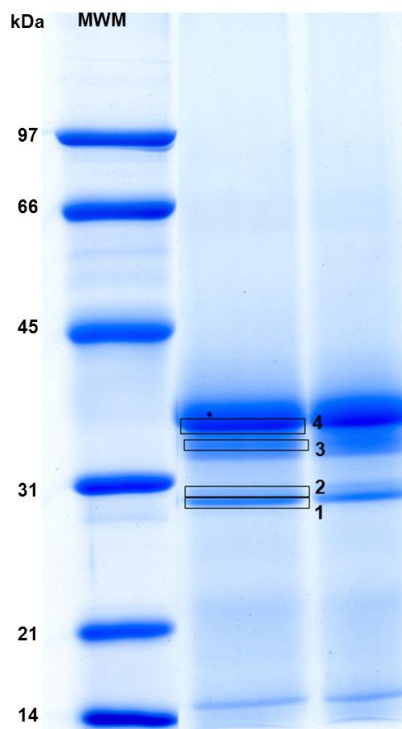


Fig. 1. SDS-PAGE of proteins extracted from *S. nigra* L. branches by PHWE at 60 °C. MWM – molecular weight marker.

Protein bands 1-4 were excised from the gel, in-gel digested with trypsin and the resulting peptides were analyzed using MALDI-TOF/TOF mass spectrometry. An example of MALDI-TOF mass spectrum of the tryptic digest of the protein band 4 is shown in Fig. 2.

Presence of individual proteins identified in the protein bands marked in Fig. 1:

- 1 ribosome-inactivating protein (*Sambucus nigra*)
- 2 ribosome-inactivating protein (*Sambucus nigra*)
- 3 ribosome-inactivating protein SNAI', nigrin b (*Sambucus nigra*)
- 4 nigrin b, ribosome-inactivating protein SNAI', ribosome- inactivating protein SNAIf precursor (*Sambucus nigra*)

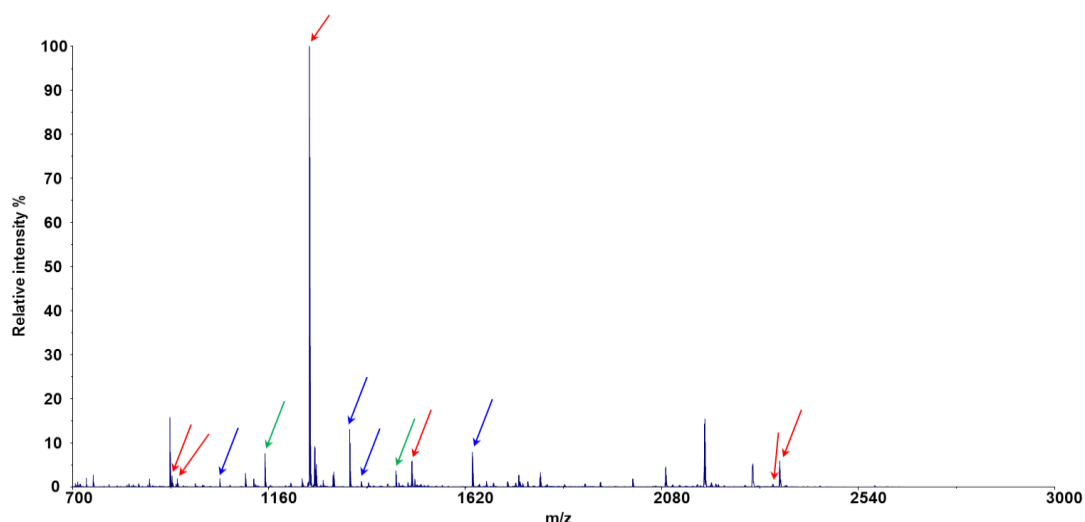


Fig. 2. Positive ion MALDI-TOF mass spectrum of the tryptic digest of the protein band 4. Denoted mass signals were identified on the basis of MS/MS analysis. Red arrows: nigrin b-related peptides; Green arrows: ribosome-inactivating protein SNAI'-related peptides; Blue arrows: ribosome-inactivating protein SNAIf precursor-related peptides.

4 Conclusions

Optimal conditions for extraction of bioactive proteins from elder branches by PHWE were found. The presence of some of these proteins was confirmed and their identity was determined by MALDI-TOF/TOF mass spectrometry.

Acknowledgement

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**P23 DETAILED ANALYSIS OF PHOSPHORYLATION OF
REGULATORY DOMAIN OF TYROSINE HYDROXYLASE**

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Summary

Human tyrosine hydroxylase 1 (hTH1) activity is regulated by phosphorylation of its N-terminus and by an interaction with regulatory 14-3-3 protein. In order to monitor structural changes within the regulatory domain of hTH1 (RD-hTH1, region of first 169 residues) caused by phosphorylation of S19 and S40 we have assigned NMR spectra by two different approaches. The non-uniform sampling approach based on sparse multidimensional Fourier transform allowed efficient acquisition of high dimensional NMR spectra [1]. Increased dimensionality (5D) provided significant speed up of backbone and side-chain assignment of the unstructured RD-hTH1 region (~70 residues). The rest (structured parts) of RD-hTH1 was assigned by conventional set of 3D NMR experiments.

We also measured kinetic rates of phosphorylation of RD-hTH1 by PKA and PRAK kinase to gain better understanding of phosphorylation process. Kinetic series were derived from intensity changes of peaks of selected residues close to phosphorylation site. The successful phosphorylation was proven by mass spectroscopy using MALDI-TOF-MS.

1 Introduction

Tyrosine hydroxylase is an enzyme catalyzing conversion of amino acid tyrosine into L-DOPA. This reaction is the initial and rate-limiting step in subsequent biosynthetic pathway leading to important catecholamine neurotransmitters -- dopamine, noradrenaline and adrenaline. The enzyme has three domains – N-terminal regulatory domain, catalytic and short C-terminal tetramerization domain. The regulatory domain can be phosphorylated and afterwards can interact with 14-3-3 protein. The interaction mode is non-trivial as there are two phosphorylation sites within RD-hTH1 and 14-3-3 proteins are dimeric, therefore having two binding sites [2].

2 Experimental

¹⁵N-RD-hTH1 and ¹³C,¹⁵N-RD-hTH1 uniformly labelled samples were prepared in our laboratory. The protein concentration was 1.0mM in 20mM sodium phosphate buffer of pH=6, 8% D₂O and 3mM sodium azide. The phosphorylation was performed in a buffer containing 50mM sodium phosphate, 10mM magnesium chloride, 0.1mM EDTA and 2mM DTT. Before NMR measurement, 8 % of D₂O and 1.5 ul of 1M sodium azide were added to the solution.

The phosphorylation was done using kinases PKA (NEB) and PRAK (University Dundee, Scotland). The concentration of PKA was 1 $\mu\text{g/ml}$ and the concentration of PRAK was 26.5 $\mu\text{g/ml}$.

NMR measurement of assignment spectra was performed using Bruker 850MHz spectrometer equipped with cryogenic triple-resonance probehead. The measurements with phosphorylated sample were done on Bruker 600MHz spectrometer with cryogenic probehead. All measurements were performed at the temperature of 293.2 K. All NMR data were processed using software nmrPipe, SPARKY and nufft package [3].

Mass spectroscopy data were acquired using MALDI-TOF-MS spectroscope.

3 Results and Discussion

In order to monitor the structural changes within the whole regulatory domain of hTH1 (RD-hTH1, region of first 169 residues) caused by phosphorylation of S40 and S19 we have assigned NMR spectra by two different approaches. The non-uniform sampling approach (NUS) based on sparse multidimensional Fourier transform allows efficient acquisition of high dimensional NMR spectra. Significant speed up was achieved in NUS NMR spectra acquisition for backbone and side-chain assignments of the unstructured region (about first 70 residues) of the RD-hTH1 thanks to the increased dimensionality (5D). The rest (structured parts) of RD-hTH1 was assigned by conventional set of 3D NMR experiments.

RD-hTH1 was phosphorylated at S40 by catalytic domain of cAMP-dependent protein kinase (PKA) with high specificity and afterwards at S19 by p38-regulated/activated protein kinase (PRAK).

The process of S19 phosphorylation by PRAK kinase was monitored using set of 29 HSQC spectra measured sequentially. Kinetic series were derived from intensity changes of peaks of selected residues close to phosphorylation site (Figure 1). The kinetic rate for concentration of kinase $c = 1 \mu\text{g/ml}$ was determined as $k = 0.11 \text{ s}^{-1}$.

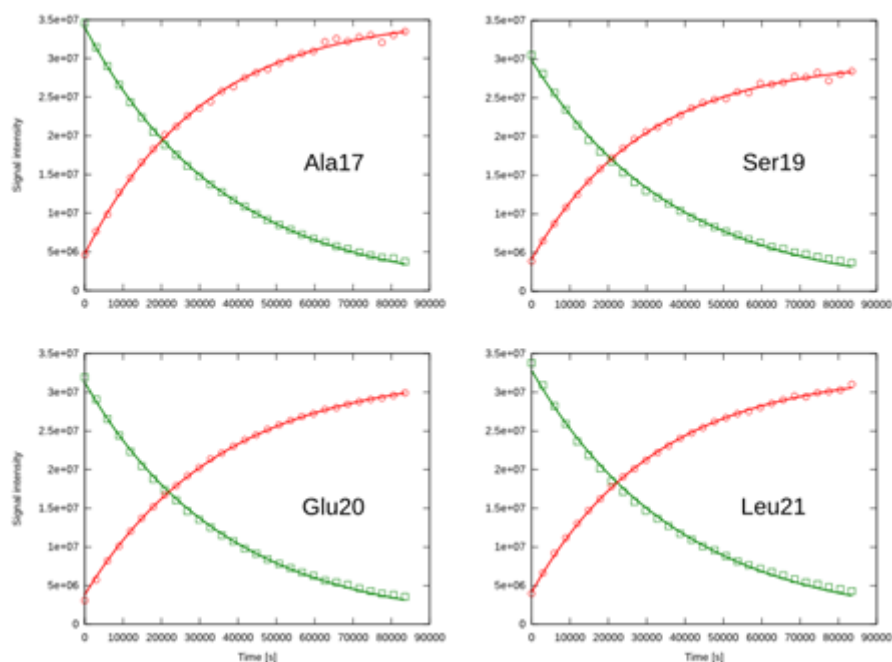


Fig. 1. Kinetics of RD_hTH1 phosphorylation at Ser19 by PRAK kinase. Kinetic curves shown only for selected residues. Intensities were fitted using exponential equations. The green points are intensities of given residues, while the red points are the intensities of signals of phosphorylated protein.

4 Conclusions

The combination of the advanced NMR and set of bioanalytical approaches deepened our understanding of the properties of regulatory domain of human tyrosine hydroxylase 1. The kinetics of phosphorylation of S19 by PRAK kinase was determined using set of 2D spectra. This approach is very general for slow processes and can be employed in other experimental systems.

Acknowledgement

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P24 ELECTROPHORETIC BEHAVIOR OF FLAVONOLIGNANS IN MILK THISTLE (*SILYBUM MARIANUM*)

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Summary

This work focuses on the development of CE method for the separation of flavonolignans in milk thistle (*Silybum marianum*).

1 Introduction

Milk thistle (*Silybum marianum*) is medicinal plant from *Asteraceae* family, which has been traditionally used for the treatment of liver dysfunction. The extract derived from the milk thistle, silymarin, is a mixture composed mostly of structurally similar flavonolignans and flavonoid taxifolin [1]. The structural formulas of flavonolignans silybin A (SBA), silybin B (SBB), isosilybin A (ISBA), isosilybin B (ISBB), silychristin (SCH), silydianin (SD) and their precursor taxifolin (TX) are depicted in Fig. 1. Two groups of natural diastereomers were identified: SBA and SBB, and ISBA and ISBB [1].

There are only two reports on the separation of flavonolignans and the quality control of silymarin extract by electromigration methods [2, 3]. Unfortunately, both CE methods fail to resolve all isomers within one run. On the other hand, LC-MS method based on RP-chromatography using C-18 column enables the separation of all main flavonolignans [4].

Therefore, the main goal of this work was to develop CE method enabling to separate all main active components in silymarin.

2 Experimental

All measurements were conducted on Agilent CE 7100 capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) equipped with a DAD detector and a 3D-CE ChemStation software (rev.B.04.03-SP1). The separation was performed in fused silica capillary of 50 µm id (48.5 cm total length and 40 cm effective length) at 25 kV

and 25°C. The analytes were detected at 200 and 320 nm. The samples were injected hydrodynamically at 50 mbar for 6 sec.

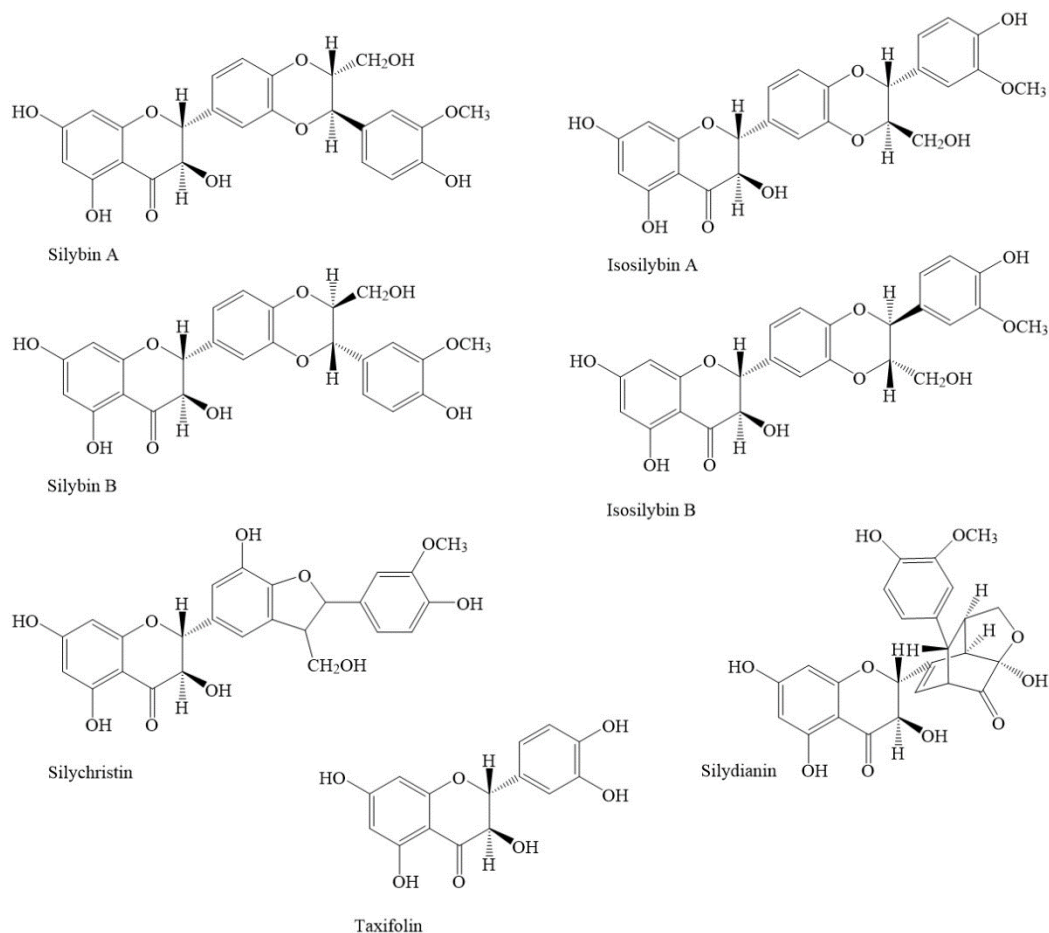


Fig. 1. Structural formulas of the main flavonolignans in *Silybum marianum* and their precursor taxifolin.

3 Results and Discussion

3.1 Electrophoretic behavior of silybin A and silybin B

Since the flavonolignans from *Silybum marianum* are structurally very similar, the method development was initially focused on the separation of diastereomers SBA and SBB. Considering the fact that according to Quaglia et al [3] the separation of SBA from SBB in borate buffer of pH 9.0 containing 12 mM β -CD was not achieved, different pseudostationary phases were tested to separate these isomers in 100 mM borate buffer (pH 9.0) containing 10% (v/v) of MeOH. Micellar electrokinetic chromatography (MEKC) based on 70, 140 and 220 mM SDS enabled the separation of silybin diastereomers with resolution (R_s) of 0.67, 1.26 and 2.00, respectively. MEKC with 120 mM deoxycholate showed worse resolution ($R_s = 0.68$), while microemulsion electrokinetic chromatography with BGE containing 140 mM SDS, 80 mM heptane, 10% (v/v) n-butanol and 100 mM borate of pH 9.0 failed in the separation of SBA and SBB. Further experiments were performed in BGE with 140 mM SDS as

a compromise between the resolution, migration time, and generated current. All flavonolignans were at least partially resolved except of ISB diastereomers migrating in a single zone.

3.2 Electrophoretic behavior of isosilybin A and isosilybin B

Surprisingly, the addition of SDS did not lead to the separation of ISB diastereomers. Therefore, the effect of β -cyclodextrin (β -CD; 0.5-15 mM) and 2-hydroxypropyl- β -CD (HP- β -CD; 0.5-5.0 mM) on the separation of ISBA and ISBB was examined. All runs were conducted in 100 mM borate buffer of pH 9.0 with 10% (v/v) of MeOH. The best resolution ($R_s = 0.86$) was achieved by the addition of 1.0 mM HP- β -CD to the BGE, while β -CD was less efficient ($R_s = 0.63$ at 1.0 mM). The addition of CDs in concentrations exceeding 1.0 mM led to worsening of resolution resulting finally in comigration of both isomers at higher concentration levels (10 and 15 mM for β -CD; 5 mM for HP- β -CD). Startlingly, no separation of SBA and SBB was observed when using β -CD or HP- β -CD as selector.

3.3 Separation of all flavonolignans by means of CD-MEKC

The above mentioned results suggest that the separation of SB isomers is affected by SDS while the resolution between the peaks of ISBA and ISBB is achieved by the addition of β -CD or HP- β -CD. As a consequence, cyclodextrin-modified MEKC (CD-MEKC) was examined to achieve the separation of all flavonolignans. Since HP- β -CD exhibited higher resolving power for ISB, the addition of this CD in the concentration range 1.5-10.0 mM was tested in the BGE consisting of 100 mM borate (pH 9.0), 140 mM SDS and 10 % (v/v) MeOH. As a compromise between the ISB resolution and CD consumption, all flavonolignans were finally separated in the BGE containing 100 mM borate (pH 9.0), 140 mM SDS, 5 mM HP- β -CD and 10 % (v/v) of MeOH (see Fig 2).

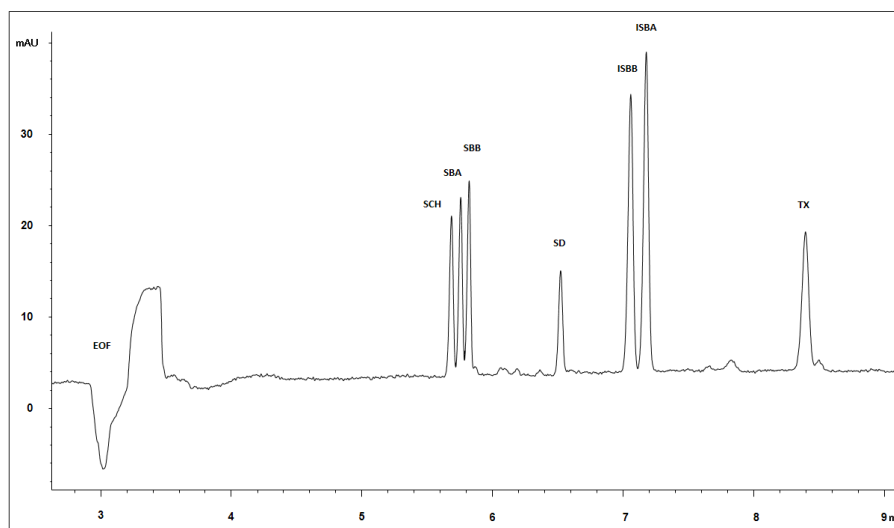


Fig. 2. Separation of flavonolignans in the BGE consisting of 100 mM borate (pH 9.0), 140 mM SDS, 5 mM HP- β -CD, 10 % (v/v) of MeOH.

4 Conclusions

The separation of diastereomers SBA and SBB was achieved by the addition of SDS as pseudostationary phase, while the separation of ISBA and ISBB was possible with addition of β -CD or 2-HP- β -CD. Since the suggested CD-MEKC conditions did not provide base-line separation of all flavonolignans, the method development will further continue with the chemometric optimization of separation conditions employing central composite design.

Acknowledgement

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P25 PREPARATION OF DOUBLE CROSS-LINKED NANOPARTICLES USABLE FOR *IN VIVO* APPLICATIONS

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Summary

Nowadays the use of drug-delivery systems becomes more and more popular. The use of supporting medium for drug substances is favored because of delayed and more controllable release. Biodegradable polymers, such as hyaluronic acid, represent the most natural materials for usage in humans. In this work we focused on multi-cross-linking of high-molecular weight hyaluronic acid (1500 – 1800 kDa) to achieve nanoparticles with hydrodynamic diameters lower than 100 nm.

1 Introduction

Nanomaterials based on biocompatible and biodegradable materials are often used as drug delivery systems [1-3]. One of these materials is hyaluronic acid (HA) which occurs naturally in human or animal body. HA can reach molecule weigh up to 8000 kDa based on number of repeating disaccharide units consisting of D-glucuronic acid and N-acety-D-glucosamine. These units are connected via beta-linkages in alternating position (1,3- and 1,4-) [4-6].

Since the hyaluronic acid was discovered, its physiological aspects were studied extensively. Thanks to its hydrophilic, moisturizing and immunosuppressive properties HA is often used in pharmaceutical industry, especially in its high-molecular weight form [7].

2 Experimental

2.1 Chemicals

Hyaluronic acid sodium salt from *Streptococcus equi* (1500 – 1800 kDa), adipic acid dihydrazide, N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC) and dialysis tubing cellulose membrane (avg. flat width 25 mm) were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetone (reagent grade) was supplied by Penta (Praha, CZ).

2.2 Preparation of hyaluronic acid nanoparticles by double cross-linking

For preparation of HA nanoparticles already published protocol with use of adipic acid dihydrazide as crosslinking agent with some modification was used [8]. Briefly, 1.36 mL of acetone was added to 2 mg of HA (2.5 mg/mL solution) and stirred for 15 min in room temperature (RT). Than the addition of 1 mg of EDAC and 1 mg of adipic acid dihydrazide followed dropwise and the mixture was stirred for 30 min in RT. Afterwards the next addition of acetone was performed stepwise in three repetitions (0.81 mL per each step) with in between 30 min incubation to reach 300/80 acetone/water ratio (w/w). Surplus acetone was then removed by evaporation and residual volume was dialyzed against deionized water overnight in RT with gentle rotation. The hydrodynamic diameter and size distribution of prepared nanoparticles were measured (Zetasizer Nano ZS instruments, Malvern Instruments Ltd., UK). Subsequently gained nanoparticles were undergone to repeat cross-linking according to protocol described above with following verification of the hydrodynamic diameter and size distribution.

2.3 Preparation of hyaluronic acid nanoparticles by simplified double cross-linking

Mixture of HA solution, EDAC and adipic acid dihydrazide were performed as described in part 2.2 until first step of acetone addition (included). Then the step with repeat cross-linking was incorporated (same amount of EDAC and adipic acid dihydrazide). After that, the protocol continued with 2nd and 3rd acetone addition (as described in part 2.2). Nanoparticles synthesis is finished by dialysis and confirmation by measurement of hydrodynamic diameter and size distribution.

3 Results and Discussion

Nanoparticles made of hyaluronic acid (HA) were prepared from high-molecular weight hyaluronic acid to obtain nanoparticles suitable for *in vivo* applications. Our goal was to prepare HA nanoparticles with hydrodynamic size lower than 100 nm which facilitate its administration, distribution in organism and influence potential cellular uptake of nanoparticles and its cytotoxicity. The main factors of cellular uptake and influence to cytotoxicity are: size and shape of nanoparticles, surface charge, hydrophobicity etc. For phagocytes the strong cytotoxic effect exhibit microparticles in contrast with nanoparticles [10]. Berkland and Fakhari (2013) discovered that nanoparticles size is depended on molecular weight of used hyaluronic acid. With increased molecular weight of used hyaluronic acid, the less size of prepared nanoparticles can be anticipated. They used as highest molecular weight 1500 kDa HA molecule and achieved nanoparticles with size about 87 ± 2.5 nm [8]. In our work we used 1500 – 1800 kDa hyaluronic acid and achieved nanoparticles with size 125.1 ± 1.802 nm in one lot and 83.12 ± 0.726 nm in second independently prepared lot. Both nanoparticles were than cross-linked again via the same protocol followed (2.2 of experimental) and the nanoparticles size was reduced to 83.12 ± 0.726 nm and 76.50 ± 0.666 nm (Table 1). Effect of double cross-linking was evaluated by measurement of hydrodynamic diameter (Figure 1). Secondly, the developed protocol was simplified with focus on decrease time of preparation (one dialysis procedure, incubations) and reagents consumption. Unfortunately, results observed by this simplified cross-linking protocol were non-measurable because of presented fiber-type structures in final product (Figure 2). This is caused by intramolecular cross-linking instead of intermolecular cross-linking preferred by increasing content of acetone to break out hydrogen bonds and due to high-molecular weight used HA [8]. This condition met the double cross-linking protocol where the addition of acetone followed to weight ratio acetone:water to 300/80. Moreover, transfer to deionized water by dialysis can stabilize natural constitution in which the HA bound large quantities of water (hydrophilic and moisturizing properties). This step seems to be necessary for further intra-molecular cross-linking.

Table 1. Hydrodynamic diameters of prepared nanoparticles by standard preparation of HA nanoparticles and by double cross-linking.

	Once cross-linked nanoparticles	Double cross-linked nanoparticles
Lot 1	$125.1 \text{ nm} \pm 1.802 \text{ nm}$	$83.12 \text{ nm} \pm 0.726 \text{ nm}$
Lot 2	$91.83 \text{ nm} \pm 0.293 \text{ nm}$	$76.50 \text{ nm} \pm 0.666 \text{ nm}$

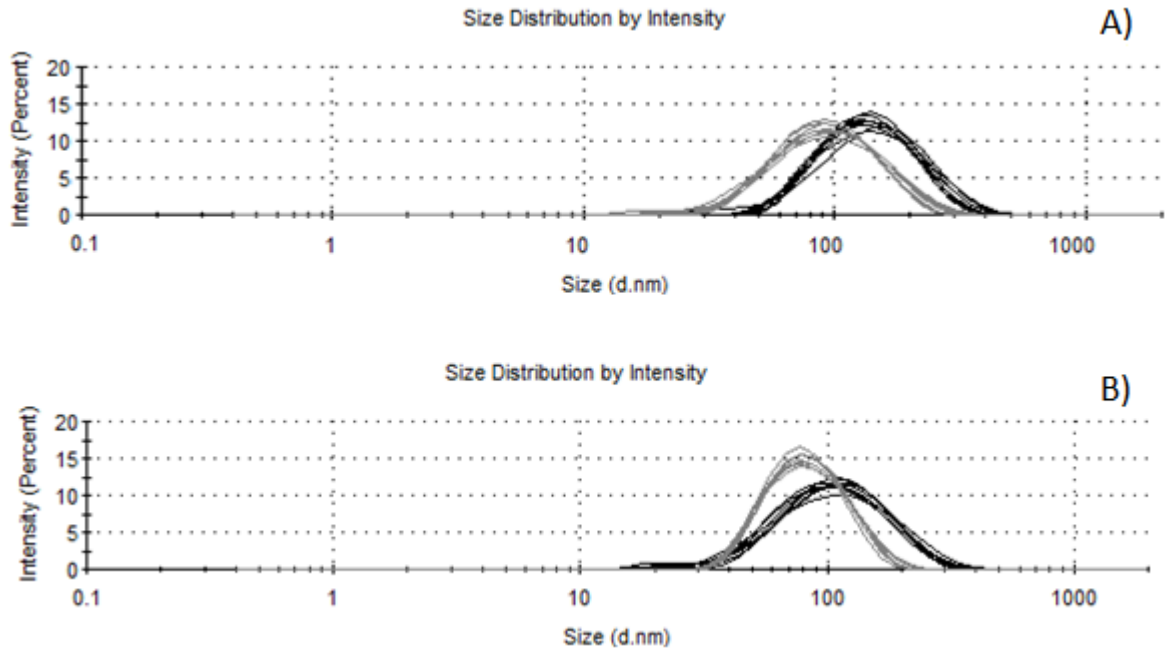


Fig. 1. Size distribution and hydrodynamic diameters measured by Zetasizer Nano ZS instruments (Malvern Instruments Ltd., UK) for lot 1 (A) and lot 2 (B). Black lines: once cross-linked HA nanoparticles, grey lines: HA nanoparticles after double cross-linking.

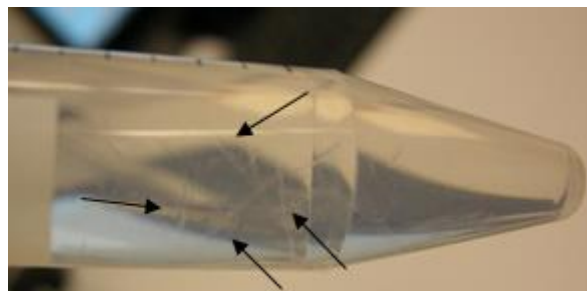


Fig. 2. Formation of fiber-type structures (arrows) during simplified double cross-linking of HA nanoparticles.

4 Conclusions

Preparation of hyaluronic acid (HA) nanoparticles was focused on multi-cross-linking protocols with goal to decreased obtained size of nanoparticles. Double cross-linking protocol was successfully developed for preparation of hyaluronic acid nanoparticles from high-molecular weight HA (1500 – 1800 kDa) with successful reducing size from 125.1 ± 1.802 nm (lot 1), resp. 83.12 ± 0.726 nm (lot 2) to 91.83 ± 0.293 nm (lot 1), resp. 76.50 ± 0.666 nm (lot 2). This brings novel approach to preparation of HA nanoparticles with sizes lower than 100 nm which are suitable for *in vivo* applications. Unfortunately, by simplified double cross-linking protocol was formed fiber-type structures instead of nanoparticles.

Acknowledgement

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P26 A COMPARISON OF X-RAY COMPUTED TOMOGRAPHY AND MAGNETIC RESONANCE IMAGING OF MOUSE BRAIN

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Summary

In this paper, we compare the use of X-ray micro computed tomography (microCT) in contrast to standardly used micro magnetic resonance imaging (microMRI) for purpose of brain tissue visualization. The possibilities of both, microCT and microMRI analysis, are demonstrated on mouse brain.

1 Introduction

X-ray computed tomography (CT) is a technology that enables to scan objects and to display its inner structures. Application of CT scanning methods are wide: from high energy CT scanners in industry, which can help show defects in various range of

materials, to applications in human and veterinarian medicine. CT imaging methods are even essential part of primary biological research where there is need to image inner structures of sample while sample needs to stay intact. We compare novelty use of X-ray computed micro tomography with enhanced contrast in imaging of mouse brain to traditional brain imaging via microMRI.

2 Experimental

First of all, the mouse brain (adult female) within the skull was measured by microMRI. Bruker Avance 9.4T MRI system (Bruker Biospin MRI, Ettlingen, Germany) was used and the results were obtained with pixel size: 0.0027cm x 0.0027 cm (matrix 512x512). Sample for MicroCT measurement was stained in 1% Iodine solution [1]. A soft tissue has a low contrast in CT data, so there is a need to enhance contrast of soft tissues with staining. MicroCT measurement were performed by GE Phoenix v|tome|x L240, equipped with 180 kV / 15 W nanofocus tube. The data was obtained with voxel resolution 6.5 μm .

3 Results and Discussion

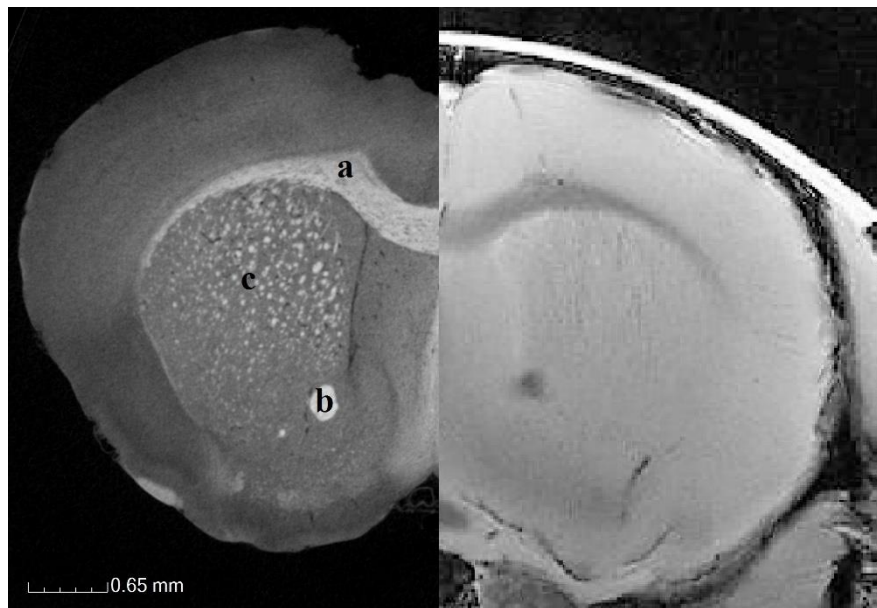


Fig. 1. MicroCT cross-section (on the left) showing areas corpus callosum (a), anterior commissure (b), and caudoputamen (c). Corresponding MicroMRI cross-section on the right.

CT imaging of brain provided images with higher resolution and contrast than images from MRI (see figure above).

4 Conclusions

The contrast enhanced micorCT measurement of mouse brain can provide images with higher resolution and higher contrast than microMRI technique. Thanks to the reached

contrast it is possible to distinguish more regions and segment them into the 3D model. The microCT measurement (2 hours) is much quicker than the microMRI measurement (10 hours). On the other hand, for the microCT measurement the brain sample had to be stained. For this purpose, the brain was separated from the skull and this manual manipulation could cause some damages. Furthermore, the staining procedure brings a certain shrinkage of the sample.

Acknowledgement

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P27 PLASMONIC ACTIVITY OF ELECTROCHEMICAL ELECTRODES MADE OF TUNGSTEN DISULFIDE NANOTUBES DECORATED WITH GOLD NANOPARTICLES

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Summary

Plasmonic metal nanoparticles have recently brought a lot of benefits to traditional branches of research including electrochemistry. Since both plasmonic and electrochemical systems' response is governed by the electron dynamics, their combination can enhance catalytic activity and bring new technological applications. In this study we investigate how the presence of tungsten disulphide nanotubes influences the catalytic activity of indium tin oxide electrodes. We demonstrate the ability to decorate the nanotubes with gold nanoparticles introducing the plasmonic feature to the system which is relevant for exploring the plasmon-enhanced electrochemistry of transition metal dichalcogenides.

1 Introduction

Electrochemistry is a well-established branch of physical chemistry which gains information about the analyte according to its ability to provide or accept electrons. Electrochemical analytical methods deliver precise information about the presence and amount of the analyte and allow fast *in situ* tracking of chemical pathways close to the electrode surface while the instrumentation is kept relatively cheap and straightforward [1]. Recently, similarly to other established fields, electrochemistry has benefitted from advances in nanotechnology. Examples include utilization of metallic nanoparticles for a detection of single base mismatches in DNA [2] or a use of carbon nanotubes to enhance the overall electrochemical signal [3].

The basis of electrochemistry is the movement of charge, therefore, additional source of charge carriers would be beneficial. One way to introduce the energetic electrons (so-called hot electrons) to the reaction is through plasmon resonance which can be generated in metal nanoparticles upon illumination with light of a certain wavelength [4]. The limit in generating hot electrons by a light from a narrow part of the visible spectra was recently overcome by Gómez et al. who have achieved a broadband activity with their gold nanoparticle assembly on TiO₂/Au support [5].

Here we introduce a study of an inherent electrochemistry of tungsten disulfide (WS₂) nanotubes and how it is influenced by a decoration with plasmonic nanoparticles. We chose WS₂ as a representative of a class of transition metal dichalcogenides [6] which have been proposed as potential candidates for electrochemical sensing [7] and photocatalysis [8].

2 Experimental

2.1 Electrode preparation

WS₂ nanotubes (WS₂ NTs), typically of 30-200 nm in diameter and 1-5 μm in length, were provided by R. Tenne (Weizmann Institute of Science, Israel). WS₂ NTs were decorated with gold nanoparticles using a procedure developed by Polyakov et. al [9]. The dry WS₂ NTs were resuspended in water using ultrasonic bath and then introduced into boiling aqueous solution of HAuCl₄. The solution boiled for additional 3 minutes, while stirred vigorously, and then cooled to the room temperature. By varying the HAuCl₄/WS₂ molar ratio the mean size of synthesized nanoparticles was controlled within the range of 5–40 nm (see Figure 1a)). Suspensions of NTs were then either spin coated or drop casted onto transparent and conductive indium tin oxide (ITO) covered glass which was used as a working electrode in following experiments.

2.2 Electrochemical analysis

The electrochemical activity of prepared electrodes was assessed by the cyclic voltammetry of 0.5 mM Fe(CN)₆^{3-/4-} in 0.1M KCl in a three electrode setup – working electrode (bare ITO/ WS₂ NTs covered ITO), reference electrode (Ag/AgCl) and auxiliary electrode (Pt wire) using PalmSens potentiostat. Voltammograms were recorded for various scan rates ranging from 5 mV/s to 1000 mV/s for bare ITO and two WS₂ NTs coverages.

3 Results and Discussion

Cyclic voltammograms of $\text{Fe}(\text{CN})_6^{3-/4-}$ were analyzed and assessed with respect to the current magnitude and potential difference between oxidation and reduction peak. ITO electrodes with different surface densities of WS_2 NTs have shown significantly enhanced electrochemical response. The presence of WS_2 NTs resulted in a substantial current magnitude increase while decreasing the potential difference between oxidation and reduction peaks which suggests an improved redox reaction reversibility as shown in Figure 1b).

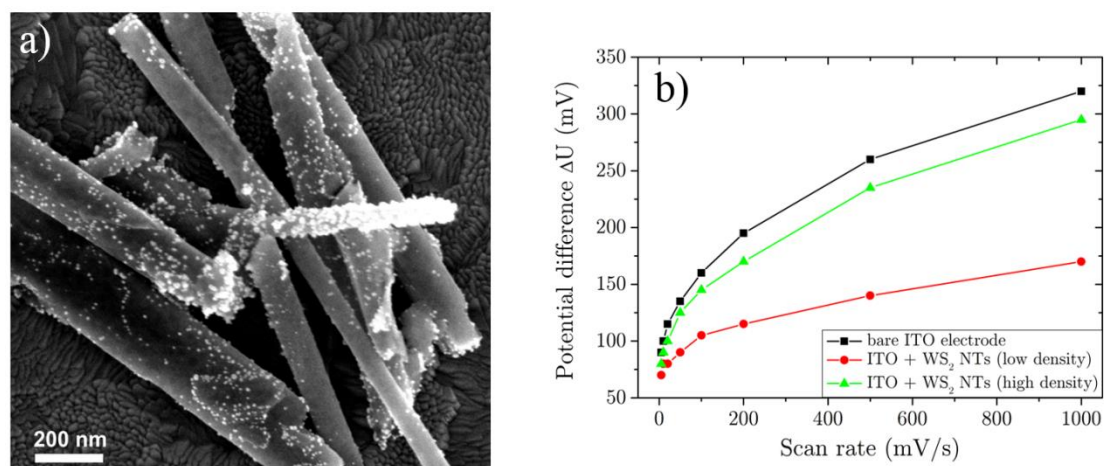


Fig. 1. a) SEM image of WS_2 nanotubes decorated with gold nanoparticles on an ITO substrate. b) Potential difference ($\Delta U = E_{p,a} - E_{p,c}$), related to the reversibility of redox reactions, between oxidation and reduction peaks of 0.5 mM potassium ferricyanide in 0.1 M KCl. The presence of bare WS_2 nanotubes improves reversibility of the reaction.

4 Conclusions

We have assessed the electrochemical properties of WS_2 nanotubes supported on transparent ITO substrate. The increasing amount of WS_2 nanotubes resulted in a significant improvement of reversibility of the tested redox reactions. We have also demonstrated the ability to decorate the WS_2 with gold nanoparticles which provide plasmonic activity to the electrode and enable us to explore the intertwined plasmonic and electrochemical effects.

Acknowledgement

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P28 CE-LIF ANALYSIS OF IMMUNOGLOBULIN G GLYCOSYATION IN MULTIPLE MYELOMA PATIENTS

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Summary

Multiple myeloma (MM) is a currently intractable malignancy of the human plasma cells. Its pathogenesis is poorly understood, and mounting evidence indicates that the bone marrow microenvironment of tumor cells has a prominent role in the malignant process [1]. It was shown that multiple myeloma (MM) IgG paraproteins exhibit unique oligosaccharide profiles [2]. In this paper we report on a novel approach for paraprotein N-glycosylation analysis from intact and papain digested molecules (Figure 1). Capillary Electrophoresis with Laser Induced Fluorescence detection (CE-LIF) was used for the analysis of the glycan patterns.

1 Experimental

Plasma IgGs and paraproteins were papain digested (Sigma-Aldrich, St Louis, MO) and purified with Protein A columns (PhyNexus, San Jose, CA). Cleavage effectivity and binding of the Fc portion was analyzed with SDS-PAGE using a Mini-PROTEAN System (Bio-Rad, Hercules, CA) by applying 120 V for 2 hours. Partitioned IgGs Fc fragments were digested with PNGase F to remove their carbohydrate moieties. The liberated glycans, the maltodextrin ladder and maltose internal standard were all fluorophore labeled by aminopyrenyltrisulfonic acid (APTS, Sciex, Brea, CA) at 37°C overnight. The unreacted fluorescent dye was removed by cleanup cartridges (ProZyme, Hayward, CA). Capillary electrophoresis profiling of the APTS-labeled N-Glycans was performed in a P/ACE MDQ automated CE instrument (Sciex) equipped with a fluorescent detector using an Ar-ion laser (excitation 488 nm, emission 520 nm).

The separations were accomplished in 50 cm effective length (60 cm total) 50 μm i.d. N-CHO capillary columns filled with the N-CHO Carbohydrate separation buffer (Sciex) by applying 500 V/cm.

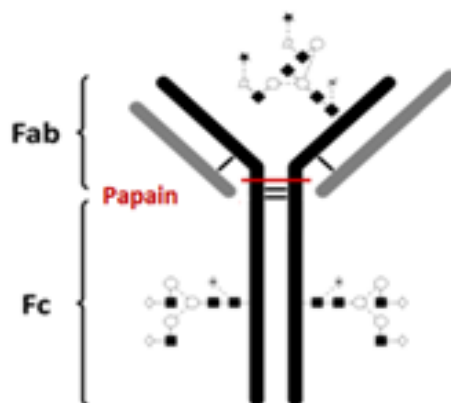


Fig. 1. Papain mediated cleavage of the IgG heavy chains. (*) shows the possible glycosylation sites.

2 Results and Discussion

Figure 2 shows the SDS-PAGE analysis of different protein A partitioned and papain digested IgG heavy chain migration of the control (lane A), untreated (lane B) and treated (lanes C, D) patient samples and non-digested human control (Lane F), untreated (lanes E) and treated (lanes G, H) serum samples. Figure 3 shows the electropherograms of total Protein A partitioned serum glycans of the normal and untreated and treated MM patients. As one can see, the normal patient sample contains all the regularly expected IgG glycans. In case of untreated MM patient samples the peaks corresponding to structures FA2G2S1, FA2, FA2G2 (peaks 10, 13 and 20) decreased. Peaks 17 and 21 corresponding to structures of FA2[3]G1 and FA2BG2 increased while some of the sialylated glycans (peaks 6, 8 and 9) disappeared. In case of treated MM patient samples, peak 13 (FA2) increased while peaks 16, 17, 18, 20 and 21 representing structures of FA2[6]G1, FA2[3]G1, FA2B[6]G1, FA2G2 and FA2BG2 decreased, while peak 19 (FA2G2) disappeared.

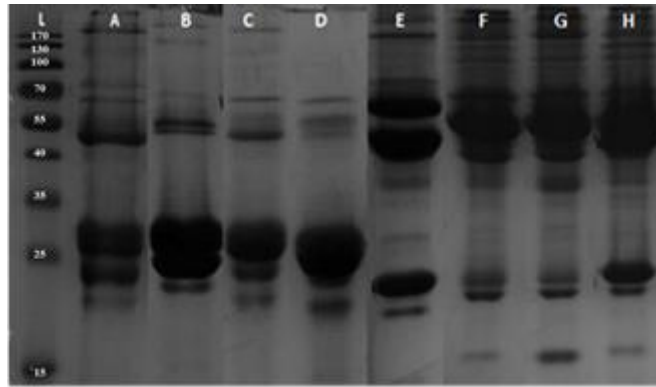


Fig. 2. SDS PAGE of papain digested Protein A bounded fraction of control and multiple myeloma (MM) patient samples. Lanes: L: protein ladder, A: normal patient sample, B: untreated patient sample, C-D: treated patient samples and native serum of control and MM patient samples. Lanes: E: untreated patient sample, F: normal patient sample, G-H: treated patient sample.

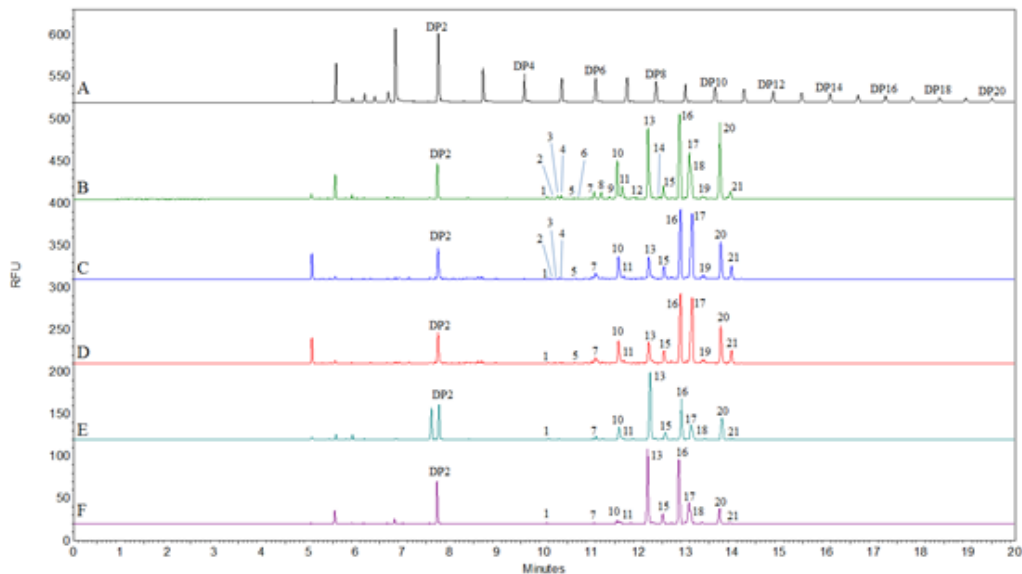


Fig. 3. CE-LIF glycosylation analysis of Protein A partitioned control as well as untreated and treated multiple myeloma (MM) patient serum. A: maltodextrin ladder, B: normal patient sample, C-D: untreated MM patient samples, E-F: treated MM patient samples.

3 Conclusions

This poster presentation reports on glycosylation pattern characterization of normal as well as untreated and treated MM patient plasma samples. Comparison of the control Protein A partitioned IgG glycans with untreated and treated MM patient samples revealed subtle differences. Based on these preliminary results, the next step will be the validation of these potential glycan biomarkers in a larger cohort.

Acknowledgement

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P29 VOLTAMMETRIC ANALYSIS OF QUANTUM DOTS-TAGGED ANTIBODIES AS A PART OF ELECTROCHEMICAL IMMUNOSENSOR ON BISMUTH FILM MODIFIED ELECTRODES

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Summary

Specific antibodies can be labelled by quantum dots (QDs) as a suitable alternative to enzymes in immunoassays. Advantages of QDs are known in the field of fluorescent immunoassays. Nevertheless, here we present the possibility of simple and highly sensitive electrochemical detection of QDs by stripping voltammetry on disposable screen printed electrodes. Moreover, modification of working electrode with in situ bismuth film could be successfully used instead of sensors with mercury film. Alpha-fetoprotein (AFP) as a protein from the group of ovarian cancer tumor markers and corresponding IgG antibodies were used as antigen-antibody system.

1 Introduction

The basic principle of the enzyme immunoassay is the specificity of the molecular recognition of antigens by antibodies to form an immunocomplex [1, 2]. Instead of less stable enzymes, quantum dots have been used as labels of biomolecules for ultrasensitive detection of biologically active molecules, and therefore we called the method quantum dot-linked immunoassay (QLISA) [3].

Quantum dots are semiconductor nanocrystals of diameters ranging from 1–20 nanometers and have been intensively studied for their fluorescent characteristics in connection to optoelectronics and biological labelling [4, 5]. The typical structure of quantum dots is core-shell where the core composed of one semiconductor (eg. CdSe, CdTe, InP, PbS) is surrounded by outer semiconductor layer (eg. ZnS) [6]. Nevertheless, QDs are usually used in conjunction with optical detection, they can be

used also in electrochemical detection where the metal component can be measured by square wave anodic stripping voltammetry (SWASV) after an acid attack, which leads to breaking the nanoparticle and releasing metal ions into the reaction solution [2,7]. Recently, the bismuth film electrodes (BiSPCEs) are used for their environmental friendliness in comparison with commonly used mercury film electrodes (MeSPCEs). The popularity of BiSPCEs consists in simple preparation, well-defined stripping signals of individual metal elements, high reproducibility [8].

2 Experimental

2.1 Apparatus

PEG-COOH capped core-type PbS QDs from Mesoligth Inc. (USA). Standard alpha-fetoprotein and polyclonal rabbit anti-AFP IgG antibodies were obtained from YO Proteins AB (Sweden). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and N-hydroxysulfosuccinimide sodium salt were provided by Sigma-Aldrich (USA). SiMAG-carboxyl magnetic particles (1 μm diameter) were produced by Chemicell (Germany).

The electrochemical measurements were performed with a portable electrochemical interface PalmSens (PalmSens, The Netherlands) controlled by software PSTrace 4.7 and connected with the three-electrode screen printed sensors with a carbon working, a platinum auxiliary and a silver pseudoreference electrode (SPCE, DropSens, Spain) and modified with in situ bismuth film (BiSPCE).

2.2 Labelling of polyclonal anti-AFP IgG antibodies by PbS quantum dots

Polyclonal anti-AFP IgG antibodies were conjugated with PbS QDs via standard one-step carbodiimide method slightly modified [9]. EDC and S-NHS (in ratio 6:1) in 0.1M phosphate buffer (pH 7.3) were added to 25 μg polyclonal anti-AFP IgG antibodies. After 10 minute preactivation at room temperature 4 μl of PbS QDs solution (8 μM) were added. After overnight incubation at 4 $^{\circ}\text{C}$ upon gentle mixing labelled anti-AFP^{PbS} IgG antibodies were affinity purified by using the antigen AFP-modified SiMAG-carboxyl magnetic particles.

2.3 Electrochemical verification of functionality of conjugate (anti-AFP^{PbS-QDs} IgG)

The functionality of conjugate labeled by QDs (anti-AFP^{PbS-QDs} IgG) was verified by using the magnetic microparticles (SiMAG-COOH, Chemicell, Germany) modified by AFP antigen. The reaction mixture of specified quantity (2.85 μl , 5.71 μl , 11.4 μl , 22.8 μl , 34.3 μl , 57.1 μl or 114.2 μl) of the conjugate (anti-AFP^{PbS-QDs} IgG) and 33,3 μg suspension of antigen modified SiMAG microparticles (corresponds to 500 ng of antigen) was incubated for 1 h at room temperature under gentle mixing in 0.1 M carbonate buffer (pH 9.4) containing 0.1 % BSA and 0.05 % Tween-20. Then it was washed three times with 0.1M phosphate buffer (pH 7.3).

Electrochemical measurement was proceeded after incubation reaction mixture (for 3 minutes) with addition of 20 μl 0.5M HCl, 70 μl 0.1M acetate buffer (pH 4.5) and 10 μl Bi³⁺ (5 mg/l) by square wave anodic stripping voltammetry (SWASV).

Addition of Bi^{3+} is achieved creation in situ bismuth film. QDs were monitored and evaluated by the recording of the peak height at potential -0.55 V. Measurement conditions were as follows: deposition potential -1.0 V for 120 s, potential range from -0.15 V to -1.0 V, step potential 0.003 V, amplitude 0.02805 V, frequency 25 Hz.

3 Results and Discussion

This work was focused on the compiling the conjugate consisting of polyclonal anti-AFP IgG antibody labelled by core type PbS quantum dots which is subsequently used as a part of QLISA based electrochemical immunosensor for quantification of AFP tumor marker exploitable for an early stage of ovarian cancer. Anti-AFP IgG^{PbS} were prepared by in-lab developed protocol due to its commercial unavailability.

Commercial PbS QDs modified by carboxy groups were chosen because of their strong and stable signal providing in square wave anodic stripping voltammetric detection (SWASV). MeSPCEs (electrode with mercury film) are usually used for analysis of heavy metals and therefore QDs. For initial testing of QDs, the MeSPCEs were used for verification the quality and response of the QDs.

For antibody labelling “in-lab” protocol was used consisting of covalent carbodiimide method of conjugation with subsequent affinity purification of gained anti-AFP IgG^{PbS} IgG antibodies by using the antigen (AFP) modified SiMAG-COOH magnetic particles. This step ensures the protection of the antibody binding site as well as purification of antibodies from unreacted free QDs.

For the verification of the functionality of the conjugate (anti-AFP IgG^{PbS}), of the antigen AFP covalently bound to the magnetic particles was done. Consequently, the resulting immunocomplex was detected electrochemically. The optimal dilution of the conjugate for next application was sought. It is important for other determining of antigen in serum. The measurement was done with screen printed electrodes with bismuth film (in situ formed), which are used because of non-toxicity and utilization for easy measurement QDs. The obtained results are shown in Fig. 1.

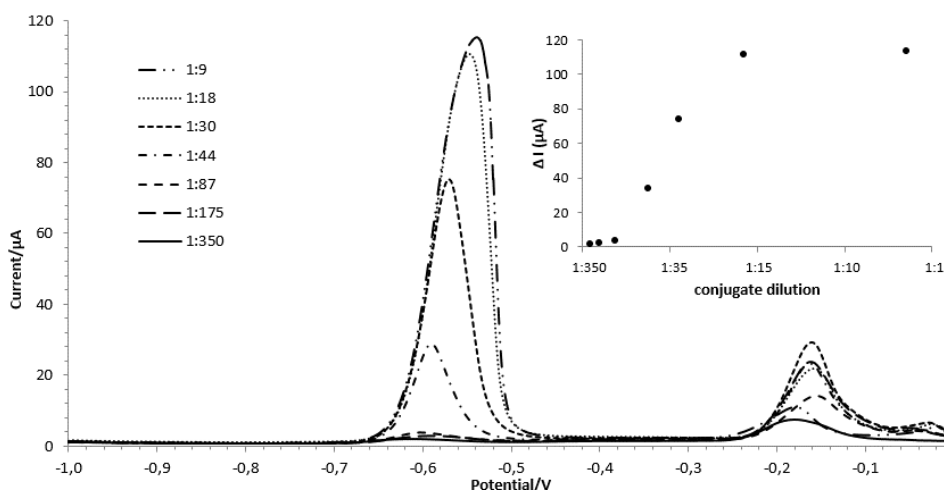


Fig. 1. Square wave voltammograms of released Pb(II) from created conjugate anti-AFP^{PbS} onto BiSPCEs (upon experimental conditions described in experimental part).

4 Conclusions

Polyclonal antibodies (anti-AFP IgG) have been successfully labeled by PbS quantum dots. Subsequently, electrochemical response of the conjugate was verified by using BiSPCEs. From these results, we can choose optimal dilution 1:18, which is usable for monitoring of the low concentration of soluble antigen AFP in serum.

Acknowledgement

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P30 DEVELOPEMENT OF MICROFLUIDIC DEVICE FOR DROPLET GENERATION AND MICROPARTICLE ENCAPSULATION

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Summary

Microfluidic devices combined with various bioanalytical and optical sensors proved their potential in chemical, biological, and biomedicine applications. At a droplet microfluidic device a fluid is divided into numerous droplets surrounded by a continuous immiscible fluid phase. Generated droplets serve then as vessels/microcapsules for delivering drugs, nutrients as well as for encapsulating of biologically active particles and cells.

Described experimental study deals with the development of a practical droplet-based microfluidic chip useful for production of monodisperse water/oil emulsion and for encapsulation of thin-metal magnetic microsheets.

1 Introduction

Microfluidic technology is a rapidly growing multidisciplinary area [1, 2]. Its advantages over conventional techniques include reduced reagent consumption, fast reaction time, and short time of analysis. Single droplets can be used for the study of many chemical systems from simple reactions immensely to complex systems, such as single cells and organelles [3].

Most of today's microfluidics tasks are based on integration of magnetic-particle manipulation technology and droplet-based devices [4]. In such systems, magnetic (nano)particles are employed as an effective and versatile tool for the isolation and detection of analytes. Moreover, surface modification of magnetic particles by specific antibodies simplifies the process of analyte extraction and their identification.

Our study reports effective droplet microfluidic system used for encapsulation of thin-metal magnetic microsheets that can be easily recognized under the microscope during their transport through the channel.

2 Experimental

The droplet-based microfluidic chip (Fig. 1) was fabricated using conventional lithography (Heidelberg μ PG 101 Laser Writer, Heidelberg Instruments Mikrotechnik GmbH, Heidelberg, Germany) on SU-8 photoresist (MicroChem Corp., Westborough, USA).

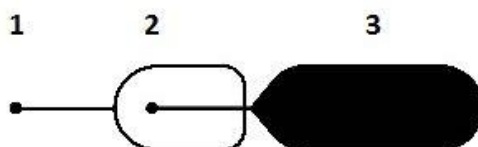


Fig. 1. Design of the microfluidic device: (1 – oil inlet, 2 – aqueous inlet, 3 – reservoir). The width of the channel was 100 μ m and the height was 25 μ m.

Commercially available Sylgard 184 polydimethylsiloxane (PDMS) kit (Dow Corning, Midland, MI, USA) containing PDMS pre-polymer and cross-linker was used in the recommended ratio of 10/1 (w/w) [5]. 3M FluorinertTM FC-40 (Sigma Aldrich) with 1% (w/w) of Pico-Surf I (Dolomite Microfluidics, UK) was injected via the oil phase inlet and aqueous phase was injected via the aqueous inlet in the ratio oil/water of 7/1 (v/v). The images of droplets were taken on a Fluorescence Lifetime Imaging Microscopy microscope (Intraco Micro spol. s r.o., CZE) equipped with XIMEA MQ013CG-E2 digital camera (ELCOM, a. s., CZE). Thin-metal magnetic circle microsheets were prepared by micro-fabrication of metal layers (Au and Ni) using lift-off photolithography [6]. Microsheets had 20 μ m in linear diameter and were 150 nm thick. There was an intent to have an effective tool for both structural binding (Au side) and magnetic properties (Ni side) in one experimental step.

3 Results and Discussion

Firstly, water droplets in fluorocarbon oil were generated under the flow rate of $70 \mu\text{L}\cdot\text{h}^{-1}$ for oil phase (OP) and $10 \mu\text{L}\cdot\text{h}^{-1}$ for aqueous phase (AP). Under those optimized conditions well-controlled production of droplet emulsion was observed (Fig. 2).

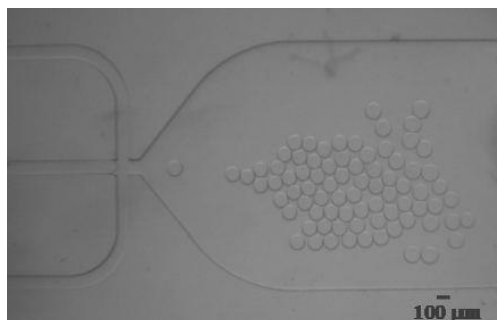


Fig. 2. View on the droplets in the reservoir of the microfluidic chip.

Secondly, we encapsulated thin-metal microsheets into produced droplets. To simplify experimental set-up, prepared microsheets were manually kept suspended in AP and their distribution into the microfluidic channel was left to be accidental and halfway uniform based on the syringe-pump pressure fluctuations. To avoid gravitational sedimentation of microsheets Hamilton syringe with AP was time to time manually overturned. Under these conditions encapsulated microsheets with random distribution were observed (Fig. 3A). During experiments, agglomeration of the microsheets as well as the sticking of the microsheets at the inlet port of AP and in the microfluidic channel was observed (Fig. 3B).

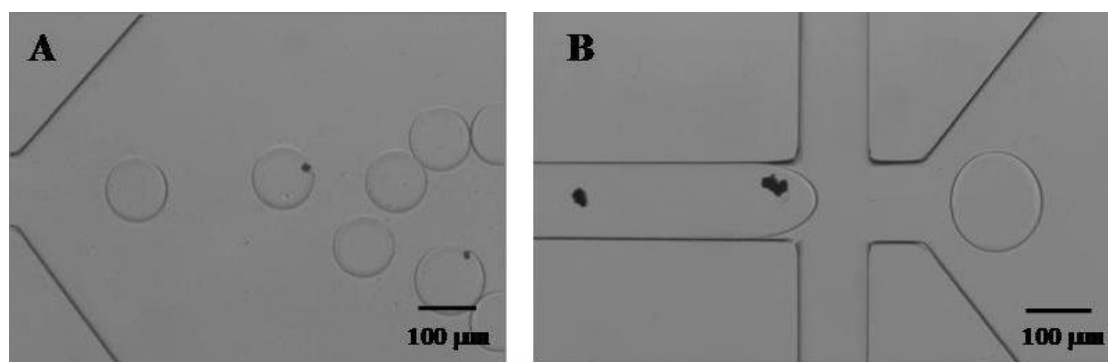


Fig. 3. Encapsulation of circle Au/Ni microsheets into droplets in the reservoir (A). Agglomeration of Au/Ni microsheets during microfluidic operation (B).

From the results, it is evident, that encapsulation ability of prepared Au/Ni microsheets is limited, depending on their size (surface to volume ratio). If the lower surface to volume ratio is used, sedimentation is much slower allowing passive encapsulation of microparticles into droplets without additional stirring. This statement

was confirmed during experiments with the well characterized spherical TiO₂ nanoparticles (TiO₂ Mag Sepharose, GE Healthcare Life Sciences).

Observed adhesion of microsheets with the channel walls and inlet ports (PDMS respectively) can be overcome by application of surfactant/polymer coating of the channel and capillary walls. Additionally, mutual interactions between microsheets can be disrupted by insertion of external magnetic field.

Performed experimental microfluidic arrangement proved its ability to produce well-controlled droplets, however direct utilization for single cell analysis still requires adjustments. Firstly, encapsulation efficiency of the metal microsheets must be increased. Secondly, it will be also important to estimate the suitable number/size of magnetic microsheets per droplets.

Acknowledgement

This work was funded by the Ministry of Education, Youth and Sports of the Czech Republic under the project CEITEC 2020(LQ1601) and by the Czech Science Foundation (P206/12/G014) and Institutional support RVO: 68081715.

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P31 DEVELOPMENT OF MAGNETIC ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF SPECIFIC ANTIBODIES AGAINST AMYLOID BETA PROTEIN AS BIOMARKER OF ALZHEIMER'S DISEASE

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Summary

The main goal of this study was to implement and optimize magnetic enzyme-linked immunosorbent assay (MELISA) method for detection of specific antibodies against

biomarkers of Alzheimer's disease (AD), specifically antibodies against beta amyloid (anti-A β). As the alternative to the traditional ELISA the surface of magnetic particles was used as solid support. In particular we focused on optimization of immunosorbent preparation and non-specific sorption minimalization. Finally, analyzed real samples confirmed the applicability of developed MELISA method.

1 Introduction

Alzheimer's disease is neurodegenerative brain disease and one of the most common causes of dementia in the elderly with an increasing prevalence and incidence in the aging population [1]. Recently, more and more studies dealing with the role of autoimmunity in the pathogenesis of AD. It was found that human IgG repertoire contains naturally occurring antibodies relating to Amyloid beta (A β), which is one of the main hallmarks of AD pathology. They may play a role in the pathogenesis as well as in the treatment of AD. That's why, development, optimization and improvements of a method, which will be suitable for detection of these antibodies is in the center of interest in this field. For the determination of antibodies, highly specific enzyme-linked immunosorbent assay (ELISA) is used. ELISA is the universal, selective and specific analytical method for detection of wide range of protein substances with the help of creation immune complexes attached to a solid phase using microtiter plate. Recently, usage of magnetic particle as a solid phase for immobilization of ligand represents an interesting immunoassay format. It offers several advantages: improvement of effectiveness of the ligand conjugation, sufficient specific surface for binding, easy handling, dispersion in whole volume and good accessibility for binding. Despite of many advantages brings this method major problem which nonspecific binding of proteins on the surface of particles responsible for high background and poor sensitivity and selectivity is. That is why effective surface modification is needed [2].

2 Experimental

2.1 Preparation of magnetic carrier with immobilized ligand

SiMAG-Amine magnetic particles (Chemicell, Berlin, Germany) were functionalized with ligands using a slightly modified 1 step or 2 step carbodiimide reaction in 0.1 M MES buffer pH 6 according to Hermanson [3]. In the case of optimizing steps 4 mg of model ligand α -chymotrypsin (α -CHT) (EC 3.4.21.1, 83,9 IU/mg, Sigma Aldrich, St. Louis, MO, USA) was immobilized by 2 step method to 1 mg of particles. For preparation of the carrier with A β (1-42) (Appronex s. r. o., Vestec, CZ) the 1 step method (100 μ g A β /1 mg of particles) was used. The immobilization efficiency was verified by standard measurement of enzymatic activity using low molecular-mass substrate N-SukcinyL-L-phenylalanin-p-nitroanilid SUPHEPA (Sigma-Aldrich, St. Louis MO, USA) in the case of α -CHT or by MicroBCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL, USA) in the case of A β peptide. Several options for blocking of surface of magnetic particles were used. Carriers with bound α -CHT were blocked with 1 % or 5% bovine albumin (BSA) (Sigma Aldrich, St. Louis, MO, USA) in 0,05 % PBS-T buffer for 1 h, room temperature or modified by

polyethyleneglycol (PEG) (Amine-PEG-carboxymethyl) (Laysan Bio, Arab, AL). Several options of modification by PEG were tested: I) PEG were immobilized on the carrier with α -CHT (0.01 M MES pH 6, 1 h, 4°C) II) PEG were co-immobilized with α -CHT together (0.01 M MES pH 6, 18 h, 4°C) III) PEG were immobilized as first and after that α -CHT were bound.

2.2 Magnetic beads enzyme-linked immunosorbent assay method for detection of specific antibodies

100 μ l of sera (1:1000 α -CHT system, 1:100 A β system) was added to 0.1 mg of magnetic particles functionalized with ligand and incubated 1 hour in 37°C under shaking. After that particles were washed 3 times with 5% BSA in PBS-T buffer. 100 μ l Goat anti-human IgG labeled with HRP diluted 1:16000 was added and incubated with particles another one hour in 37°C under shaking. Washing 3 times with 5% BSA in PBS-T buffer and 2 times with PBS buffer followed. 100 μ l of substrate solution with OPD as chromogen was added and incubated 15 min at 37°C in dark. The reaction was stopped by adding 50 μ l 1M H₂SO₄ and the absorbance was measured spectrophotometrically at 492 nm. In the same manner classical ELISA in microtitre plate to compare the sensitivity of both methods was performed.

3 Results and Discussion

In the frame of optimization of preparation suitable magnetic carrier, several types of surface modification were tested. Except classical blocking by BSA surface modification by PEG was tested. The big advantage of PEG surface modification is low molecular weight of PEG (3.4 kDa) supposing no steric hindrance. In the figure 1 summary results comparing non modified particles with various types of surface modification are shown. Although increasing BSA concentration leads to a reduction of non-specific sorption, PEG surface modification is clearly the most effective. If 2-step carbodiimide method of binding with subsequent PEGylation is used, we observed highest efficiency of binding (around 52 μ g per 1 mg of particles) together with lowest non-specific sorption expressed by absorbance value 0.16 (A 492 nm).

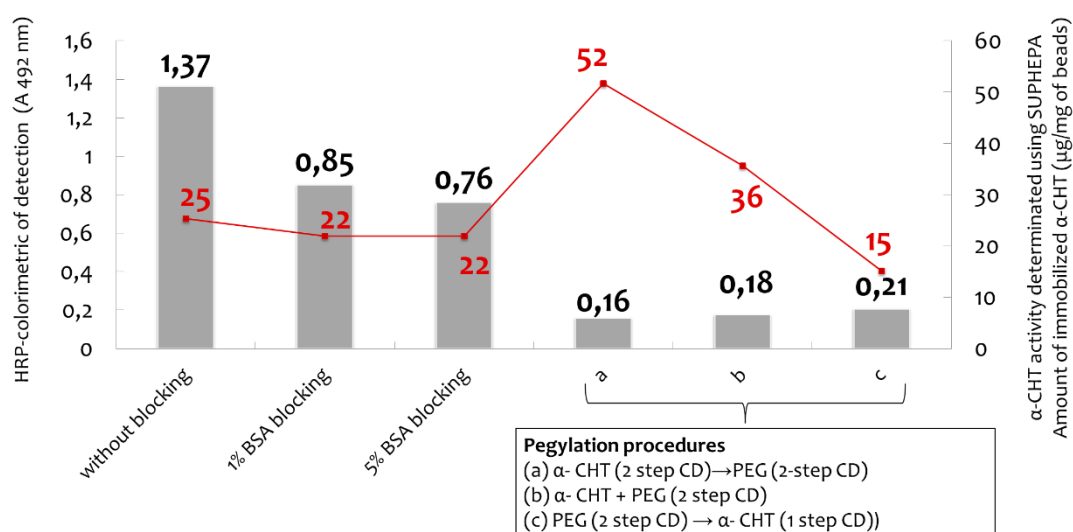


Fig. 1. Effect of beads surface modification.

Based on these results magnetic carrier with A β (1-42) was prepared. Capture efficiency was around 94% and non-specific sorption expressed by absorbance value was 0,205 (A 492 nm).

Next step was to verify the suitability of this magnetic carrier for quantification of anti-A β antibodies from human sera representing the complex biological matrix. Levels of serum anti-A β were measured in 3 patients and one healthy control sample. Finally the results measured by magnetic-beads ELISA were compared with classical ELISA in microtiter plate. As shown in the table 1 in AD patients sera are lower levels of anti-A β in comparison with healthy control. This phenomenon is in accordance with literature [4]. Higher amount of serum anti-A β detected using magnetic beads-ELISA in comparison with standard ELISA points to higher sensitivity when magnetic particles as solid phase are used.

Table 1. Quantification of anti-A β antibodies in human sera. Comparison of classical microplate ELISA and Magnetic beads ELISA. Antigen amount 15 µg, conjugate dilution 1:16000, Calibrator: mouse monoclonal anti-A β (6E10) IgG1 antibody.

	Microplate-ELISA		Magnetic beads-ELISA	
	A (492 nm)	c (ng/ml)	A (492 nm)	c (ng/ml)
Control serum (1:100)	0.669	79.1	1.793	137.6
Patient serum 1 (AD) (1:100)	0.317	36.3	1.461	111.7
Patient serum 2 (AD) (1:100)	0.387	44.8	1.655	126.9
Patient serum 3 (AD) (1:100)	0.191	20.9	1.615	123.7

4 Conclusions

In conclusion, we report that modification of magnetic particles by polyethyleneglycol considerably improves properties of magnetic carrier used as a solid phase in ELISA method. If the surface of magnetic particles is blocked by PEG nonspecific sorption of other proteins from sera is significantly decrease resulting in higher sensitivity and selectivity. Suitability of this magnetic carrier for quantification of antibodies from human sera was verified. Based on these promising results this method seems to be convenient in diagnosis of Alzheimer's disease. And due to magnetic properties of particles it could be automatized and carried out in microfluidic format.

Acknowledgement

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P32 ORGANIC MARKERS AND COMPOUNDS IN PM1 AEROSOL IN SMALL TOWN NEAR PRAGUE (CZECH REPUBLIC) IN WINTER 2016

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Summary

Contribution summarizes the concentrations of monosaccharide anhydrides, resin acids, methoxyphenols, monosaccharides, disaccharides, sugar alcohols, alkanes, hopanes, steranes and polycyclic aromatic hydrocarbons in PM1 aerosols in small town Kladno-Švermov in winter. According to concentrations of organic markers, the identification of emission sources in Kladno-Švermov is discussed.

1 Introduction

Atmospheric particulate matter (PM) is known to play an important role in many environmental problems. During last years, much attention has been paid to the identification of emission sources of PM. To track the contributions of the main sources to composition of atmospheric aerosols, various source-specific organic tracers are analysed in collected PM [1-4].

2 Experimental

2.1 Aerosol sampling

Atmospheric aerosols in the size fraction PM₁ were sampled over 24-h periods using a high-volume sampler (DHA-80, Digital, 30 m³.h⁻¹) on quartz filters during winter period of 2016 (2. 2. – 1. 3.) in the town Kladno-Švermov. Sampling locality was situated in suburban residential area.

2.2 Sample preparation, extraction and analysis

Collected aerosols were analysed for monosaccharide anhydrides (MAs), resin acids (RAs), methoxyphenols (MPs), saccharides, alkanes, hopanes/steranes (H/S) and polycyclic aromatic hydrocarbons (PAHs). Analysis of MAs, RAs, MPs and saccharides included extraction of parts of filters with mixture dichloromethane/methanol (1:1 v/v) under ultrasonic agitation, derivatization of extracts with mixture of BSTFA + TMCS, dryness, redissolution in hexane and GC-MS analysis. Analysis of alkanes, H/S and PAHs included extraction of parts of filters with mixture of dichloromethane/hexane (1:1 v/v), fractionation on column with silicagel, dryness to 1 mL and GC-MS analysis.

3 Results and Discussion

The average concentration of sum of PM₁ was 18.8 µg.m⁻³ (4.34 – 48.3). Analysed organic compounds had, in general, similar trend as the PM₁ aerosols (Fig. 1).

Presence of monosaccharide anhydrides in collected aerosols proves contribution of biomass burning to the composition of aerosols. Methoxyphenols, resin acids and retene indicate burning of wood, probably in the frame of residential heating.

Presence of hopanes, steranes and phytane and pristane in aerosols collected in Kladno-Švermov proves contribution of emissions from traffic to aerosol composition. $\alpha\alpha\alpha(20R)$ -Cholestane, phytane and pristane serve as specific markers of traffic while hopanes also serve as organic markers for fossil fuel combustion. Homohopane index [S/(S+R) = 0.09 – 0.12] and presence of picene indicate combustion of coal (brown coal) as the emission source of aerosols.

The average concentration of sum of PAHs was 28.6 ng.m⁻³ (3.50 – 105). The ratio BeP/(BeP+BaP) in all aerosol samples from Kladno-Švermov was less than 0.5 indicating emission of aerosols from local sources.

A carbon preference index (CPI) was in the range 0.84 – 1.18, which indicates vehicular emissions and other human activities as emission source of aerosols collected in Kladno-Švermov.

Analyzed saccharides prove burning of biomass as emission source but they also indicate other emission sources of aerosols such as resuspension of soil as well different biological sources (spores, pollens, fungi, microorganism metabolites, fragments of plants).

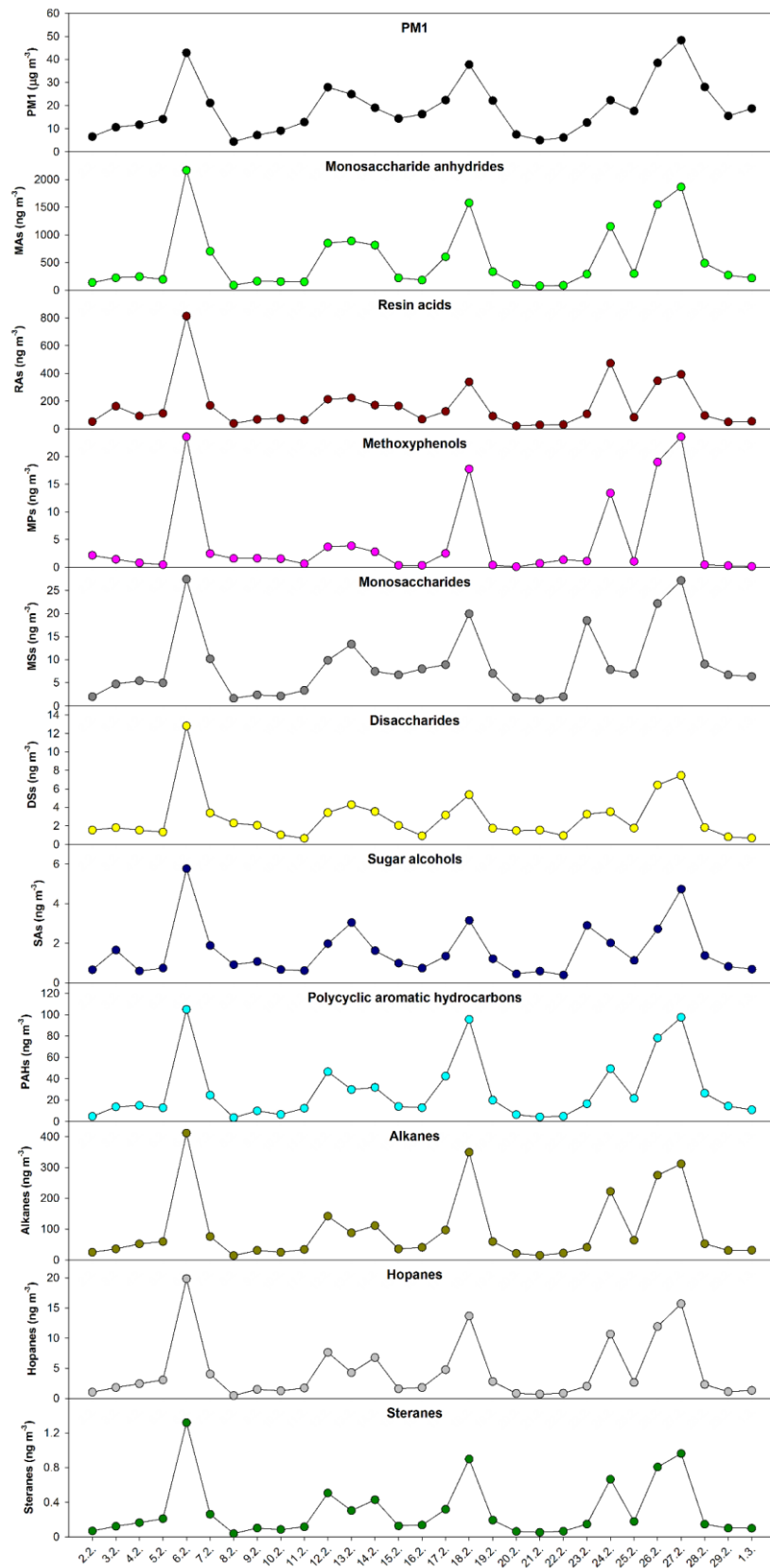


Fig. 1. Trends of mass concentrations of PM1 ($\mu\text{g}\cdot\text{m}^{-3}$) and sums of analysed organic compounds ($\text{ng}\cdot\text{m}^{-3}$) in Kladno-Švermov.

4 Conclusions

Combustion of biomass (especially soft wood) and coal and traffic belong to the biggest emission sources of organic compounds bound to aerosols collected during winter campaign in Kladno-Švermov.

Acknowledgement

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P33 MICRO-ELECTROMEMBRANE EXTRACTIONS ACROSS MULTIPLE AQUEOUS AND ORGANIC PHASES FOR SELECTIVE PRETREATMENT OF RAW BIOLOGICAL SAMPLES

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Summary

Micro-electromembrane extraction (μ -EME) across multiple aqueous and organic solutions was developed for selective extractions of various analytes from untreated biological samples. The novel approach requires μ L volumes of samples and organic solvents, which form consecutive immiscible plugs in a transparent polymeric capillary and the extraction process is accelerated by application of d.c. voltage. Number of the plugs, their composition, volume and sequence in the extraction capillary can be chosen specifically for each particular application and high variability and selectivity of the extraction process can be achieved. In this contribution, we demonstrate suitability of a five-phase μ -EME system for simultaneous extractions of cations and anions from urine and for selective extractions of basic analytes based on their acid-base strength from plasma.

1 Introduction

Miniaturized sample pretreatment techniques, such as electromembrane extraction (EME) [1], have become increasingly popular recently. EME is based on transfer of charged analytes from one aqueous phase across a thin layer of a water immiscible phase into another aqueous phase by the action of electric field. Further miniaturization of the original EME concept, so called micro-electromembrane extraction (μ -EME), was presented recently [2]. In μ -EME, a narrow plug of an organic solvent is sandwiched between two plugs of aqueous solutions in a low-bore polymeric capillary and resides there as a free liquid membrane (FLM). In this contribution, a new architecture of five-phase μ -EMEs is presented, which uses a set of multiple plugs of immiscible aqueous (one donor and two acceptor) and organic (two FLMs) solutions in a single microextraction tube.

2 Experimental

μ -EMEs were carried out in chemically inert perfluoroalkoxy (PFA) tubing (1.0/1.6 mm ID/OD, Vici-Jour, Schenk, Switzerland). PFA microextraction units were scalpel-cut to desired lengths (6-12 mm) from the tubing and were filled with appropriate volumes of aqueous solutions and organic solvents. Two platinum electrodes (0.25 mm thick tubular wires, 99.95%, Advent, Oxford, England) and a d.c. power supply ES 0300-0.45 from Delta Elektronika (Zierikzee, Netherlands) were used for all μ -EMEs.

3 Results and Discussion

3.1 Simultaneous μ -EMEs of cations and anions

The proof of principle was examined on simultaneous μ -EMEs of cationic (phenosafranine) and anionic (SPADNS) dyes. μ -EME system at three different extraction times is depicted in Figure 1. Before extraction, both dyes were present in the center of the μ -EME system in donor solution. On application of d.c. potential, the positively and negatively charged analytes migrated towards cathode and anode, respectively, and complete μ -EME of the two dyes was observed after 5 min.

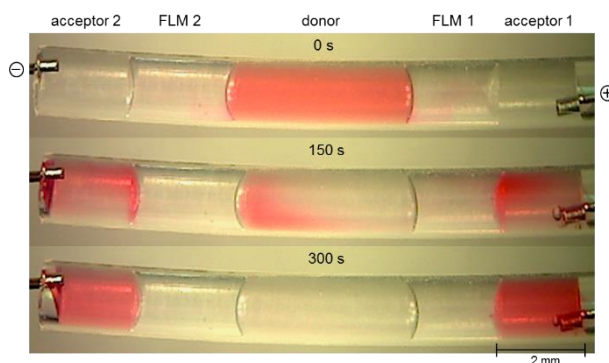


Fig. 1. Simultaneous μ -EME of cations and anions. Acceptor 1 (DI water, 1.5 μ L); FLM 1 (1-pentanol, 1.5 μ L), donor (100 μ M SPADNS/phenosafranine in DI water, 3 μ L); FLM 2 (1-pentanol, 1.5 μ L); acceptor 2 (DI water, 1.5 μ L); extraction voltage, 200 V.

Simultaneous μ -EMEs were further performed with cationic (procaine) and anionic (ibuprofen) drugs. For μ -EMEs of the drugs, FLMs and aqueous solutions were selected according to the particular extraction processes and each phase had unique composition. Simultaneous extractions of procaine (into acceptor 2) and ibuprofen (into acceptor 1) from spiked urine are shown in Figure 2 and confirm compatibility of the EME process with raw body fluids.

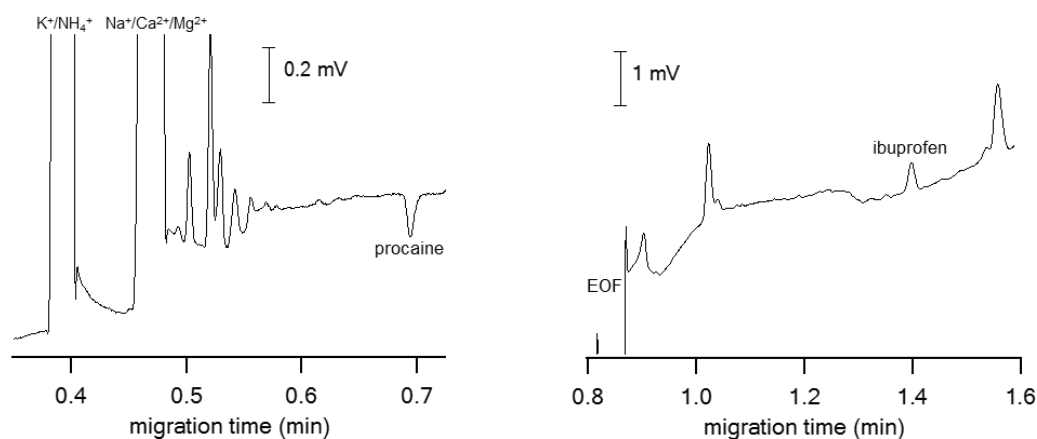


Fig. 2. Simultaneous extractions of basic and acidic drugs from urine. Acceptor 1 (2 mM NaOH, 1.5 μ L); FLM 1 (1-octanol, 1.5 μ L), donor (urine spiked with 10 mg/L of procaine and ibuprofen, 1.5 μ L); FLM 2 (ENB, 1.5 μ L); acceptor 2 (DI water, 1.5 μ L); extraction voltage, 300 V; extraction time, 5 min.

3.2. Selective μ -EMEs of basic drugs based on their acid-base strength

μ -EMEs across multiple aqueous and organic phases were further examined for selective extractions of basic drugs based on their acid-base strength. Experimental set-up for the selective μ -EMEs of basic drugs is depicted in Figure 3.

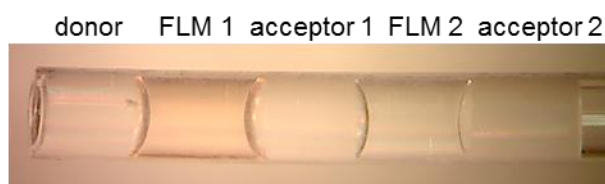


Fig. 3. Photograph of five immiscible phases formed in a PFA tubing.

Human plasma was spiked with papaverine (pK_a 6) and nortriptyline (pK_a 10.5) and selective μ -EMEs were achieved by tuning pH of acceptor solution 1 and 2. For acidic acceptor solution 1 and neutral acceptor solution 2, fractionation of nortriptyline into acceptor solution 2 and of papaverine into acceptor solution 1 was achieved (see Figure 4). Partial transfer of nortriptyline into acceptor solution 1 is subject of a further study.

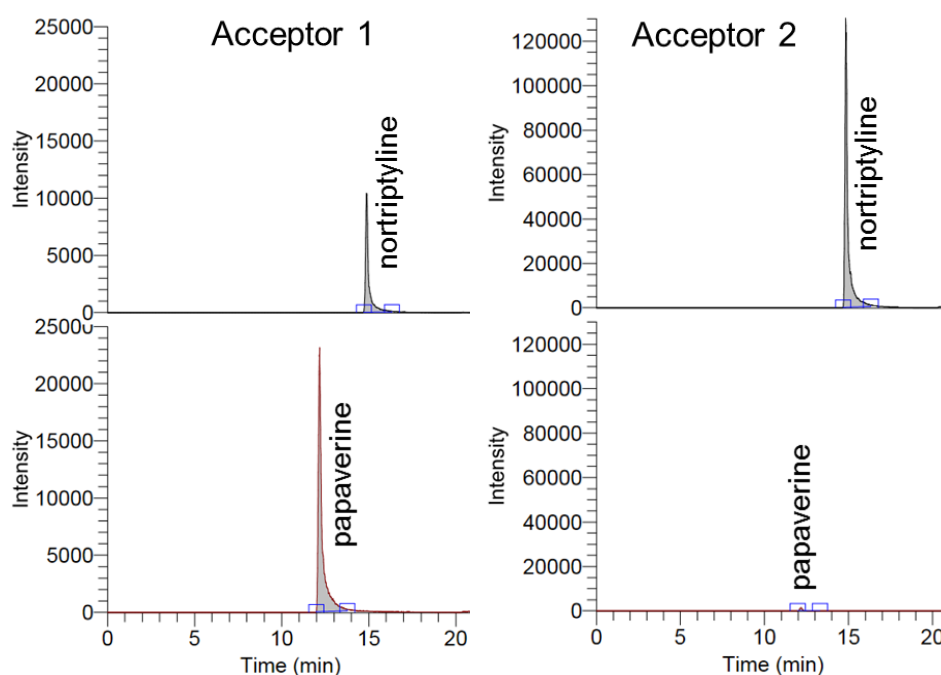


Fig. 4. Selective five-phase μ -EMEs of basic drugs. μ -EME conditions: donor (plasma diluted 1:1 with 20 mM HCl spiked with 20 mg/L nortriptyline and papaverine, 1.0 μ L), FLM 1 (4-NC, 1.0 μ L), acceptor 1 (10 mM HCl, 1.0 μ L), FLM 2 (4-NC, 1.0 μ L) and acceptor 2 (5 mM phosphate buffer at pH 6.8, 1.0 μ L). Voltage, 300 V; time, 15 min.

4 Conclusions

Selectivity of μ -EMEs can be efficiently tuned by using multiple organic and aqueous phases. Adjacent plugs of immiscible phases can be formed in transparent capillary tubing and ensure selective μ -EMEs of cations and anions as well as of compounds with same charge but different acid-base strength. As the number of the plugs, their composition, volume and sequence in the capillary tubing can be chosen specifically for each particular application, highly variable and selective extraction systems can be achieved.

Acknowledgement

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**P34 BIOBANKING OF PATIENT SAMPLES FOR GENO –
GLYCOMIC LUNG DISEASE BIOMARKER STUDIES**

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Summary

Comprehensive geno – glycomic studies to identify new tumor biomarkers depend on appropriate collection and registration of biological materials, included tissues, blood samples or other body fluids from a large number of patients and healthy individuals with associated well- documented clinical information from the sample donors [1].

1 Introduction

The aim of the project is biological sample collection (biobanking), which involves a group of lung cancer (LC), COPD and LC+COPD patients, as well as healthy controls with the necessary TUKEB permission and informed patient consent.

2 Experimental

The sample groups consist of approximately 300 LC (adenocarcinoma), 300 COPD, 300 LC+COPD, as well as 300 non – cancer related control samples. One of the most important part of our biobanking workflow is the clinical data collection from each patient (age, smoking habits, occupation, morbidity and co-morbidity, tumor/COPD stage, histology, TNM chemotherapies, surgeries, etc.) from MedWorkS information system and fill out the relevant questionnaires. The collected samples are stored at -80 °C until processed.

3 Results and Discussion

The high number of patient samples (biobank) will allow thorough geno - glycomic characterization of the four patient classes with high statistical value including SNP study, N-glycan site specificity analysis as well as quantitation of glycan linkage and positional isomers at the specific sites.

4 Conclusions

In summary, this novel combination of genetic and glycomic approaches will provide comprehensive information for both sides of the problem in hand and will lead to the

identification of disease specific glycosylation (site and microheterogeneity) changes of acute phase proteins and similarly reveal changes in glycosylation of some known cancer biomarkers.

Acknowledgement

The authors acknowledge the support of the MTA-PE Translation Glycomics Grant (#97101) and NKFIH (#K116263) grants of the Hungarian Government.

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P35 NOVEL, VERSATILE CAPILLARY ELECTROPHORESIS INSTRUMENT WITH LASER INDUCED FLUORESCENCE FOR ANALYSIS OF VARIOUS LIPID PEROXIDATION BIOMARKERS

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Summary

End product of oxidative degradation of lipids, namely malondialdehyde (MDA) and 4-hydroxynonenal (HNE), have an important role in diagnostics as biomarkers of lipid peroxidation. In this work we have developed a new sensitive method for quantitation of MDA in biological samples, such as exhaled breath condensate (EBC) and plasma. For the first time it was possible to detect 1.7 nM concentration of MDA in EBC samples by CE.

1 Introduction

Lipid peroxidation can be described as oxidative degradation of lipids, especially where free radicals attack polyunsaturated fatty acids in cell membranes, resulting in cell damage. The end products of lipid peroxidation are reactive aldehydes, such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE). MDA and HNE have cytotoxic role inhibiting gene expression and promoting cell death, hence they are bioactive markers of lipid peroxidation. They are often present in very low concentrations [1].

In this work the reaction conditions for MDA reaction with thiobarbituric acid (TBA) were optimized, such as reaction time, reaction temperature, ratio of reagent to analyte and pH of reaction solution. The CE separation conditions (BGE, pH) were

optimized for sensitive MDA analysis and very low levels of MDA in biological samples, i.e. exhaled breath condensate (EBC) and plasma, were determined.

2 Experimental

2.1 Electrophoretis system

Capillary electrophoresis with laser induced fluorescence (CE-LIF) offers an ideal combination for the analysis of low concentrations of analytes in small volumes of biological samples. In this work, we have built a versatile CE-LIF system that allows easy exchange of different laser modules. A 532 nm green laser module was incorporated in the described system as a light source having a compatible wavelength with the highly fluorescent product of the reaction between MDA and TBA. A fused-silica separation capillary with 50 μm ID, 375 μm OD, 60 cm total length (Microquartz GmbH, Munich, Germany) was used. Separation voltage was 15 kV. The optimized BGE consisted of 50 mM borate buffer, adjusted with NaOH to pH 9.

2.2 Sampling

The EBC samples were obtained from volunteers at Masaryk University by using a small sampling device [2]. Samples were derivatized immediately after the collection and stored at $-45\text{ }^{\circ}\text{C}$. A plasma sample was treated with acetonitrile and centrifuged at 3000xg for 5min to remove proteins. Supernatant was diluted and derivatized immediately after the collection and then stored at $-45\text{ }^{\circ}\text{C}$ [3].

3 Results and Discussion

3.1 Derivatization of MDA

MDA is the most frequently studied biomarker of lipid peroxidation. The common method for analysis of MDA is by fluorescence using the thiobarbituric acid (TBA) assay. The resulting MDA-TBA product is highly fluorescent (excitation 532 nm, emission 550 nm) and negatively charged, while the unbound TBA doesn't fluoresce. The derivatization conditions were studied. The larger the excess of TBA, the faster is the reaction. As an optimum temperature of $90\text{ }^{\circ}\text{C}$ was selected. In an acidic medium ($\text{pH} < 4.5$) MDA will predominantly be in the non-dissociated enol form which readily reacts with TBA. Therefore, pH 2 was chosen as an optimum for maximum yield of the reaction with an acceptable reaction time. The reaction time of TBA with MDA was optimized. Fig. 1 shows that even at low concentrations of MDA, that are commonly found in EBC, MDA reacts with TBA in 60 minutes.

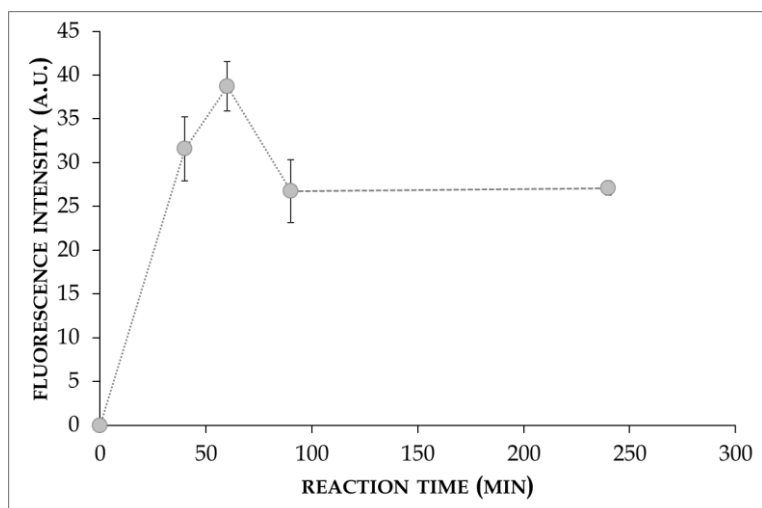


Fig. 1. Graph showing the dependence of reaction time of fluorescence intensity for 56 nM MDA solution.

3.2 Separation conditions

The type of background electrolyte (BGE), its pH and concentration significantly influence the separation efficiency (N) and peak symmetry in CE. The optimization of several BGEs and pH was carried out (results not shown). Fig. 2 shows an optimization of BGE concentration based on two factors, separation efficiency (N) and peak symmetry. In order to obtain a good separation and sharp peak shape a 50 mM borate buffer was selected as BGE.

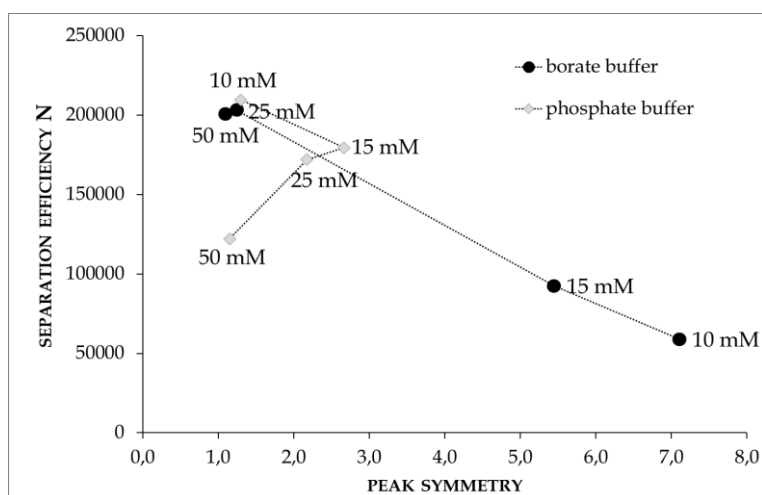


Fig. 2. Graph showing the dependence of separation efficiency N on peak symmetry for borate (pH 9) and phosphate (pH 8) buffer for optimal CE separation.

3.3 Real sample analysis

The applicability of the method has been demonstrated on the analysis of real biological samples, such as exhaled breath condensate (EBC) and plasma, and can be used also for other type samples. Limits of detection and quantification of the developed CE

method were in the nM range (1.7 nM; 8.4 nM) and are sufficient to determine MDA in real samples, such as EBC (Fig. 3) and plasma (Fig. 4).

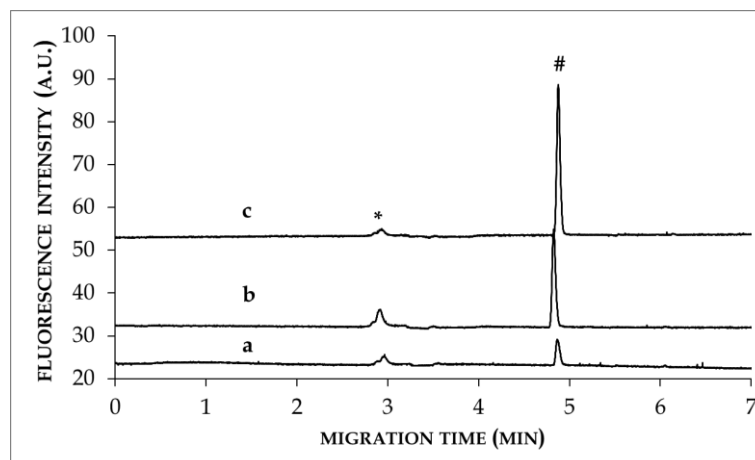


Fig. 3. Electropherograms of EBC derivatized by TBA (a) and with standard addition of 115 nM MDA (b) and 230 nM MDA (c). CE conditions: 15 kV, 50 mM BORATE buffer (pH=9), 60s hydrodynamic injection. Peak identification: * - hydrolysis of TBA, # - peak of TBA-MDA complex.

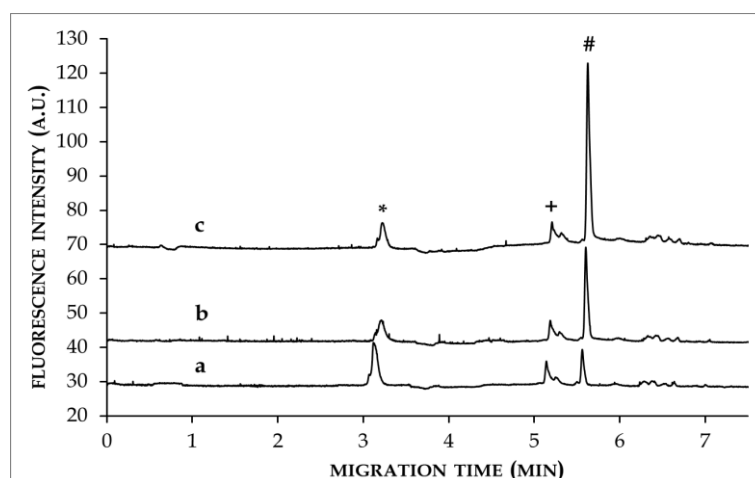


Fig. 4. Electropherograms of 10 times diluted plasma derivatized by TBA (a) and with standard addition of 115 nM MDA (b) and 230 nM MDA (c). CE conditions: 15 kV, 50 mM BORATE buffer (pH=9), 60s hydrodynamic injection. Peak identification: *- hydrolysis of TBA, + - unidentified peaks, # - peak of TBA-MDA complex.

4 Conclusions

In this work we have developed a new sensitive method for quantitation of MDA in biological samples by CE-LIF with LOD about 1.7 nM. First part of the work was devoted to the construction of a new CE-LIF system. Then the derivatization process of MDA by TBA was optimized. The ability to use this method for the detection of MDA in a biological samples, such as EBC and plasma, was also demonstrated. For the

first time it was possible to detect MDA in EBC samples by CE. Other biomarkers are now being studied.

Acknowledgement

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P36 SUBSIDIARY REAGENS FOR IMPROVEMENT OF ENZYMATIC DIGESTION PROTOCOL

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Summary

In this report, we demonstrate the influence of hydrogen peroxide, phenol and acetonitrile on enzymatic digestion of proteins accomplished during common bottom-up proteomic identifications.

1 Introduction

Enzymatic digestion of proteins is a critical step of analytical proteomics. Conventional in-gel digestion protocol includes excision of protein bands from polyacrylamide gels, washing of the gel pieces, reduction and alkylation of proteins in the gel, overnight in-gel enzymatic digestion and extraction of obtained peptides. The whole procedure consumes minimally two days. In order to accelerate and increase efficiency of proteolytic digestion, alternative methods have been developed and tested [1, 2]; *e.g.*, approaches based on microwave [3], ultrasound and infrared technology [4], on addition of water-miscible organic solvents (acetonitrile, acetone, propanol) [5, 6], or on the use of immobilized enzyme microreactors [7].

This work is focused on the investigation of a possible positive influence of the certain subsidiary agents – hydrogen peroxide, phenol and acetonitrile on tryptic digestion of several model proteins.

2 Experimental

2.1 Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Fluka (Buchs, Switzerland), except chemicals for SDS-PAGE, which were obtained from Bio-Rad (Philadelphia, PA, USA). Trypsin (sequencing grade from bovine pancreas) was obtained from Roche Diagnostics (Mannheim, Germany). ZipTip C18 pipette tips were purchased from Merck (Prague, CZ). MALDI matrix for MS analysis was bought from Laser-Bio Labs (Sophia-Antipolis Cedex, France).

2.2 In-gel enzymatic digestion

Protein solution (app. 0.7 µg of each protein) was loaded onto the 12% SDS gel. Their visualization after 1-D GE analysis was carried out using Coomassie Brilliant Blue G-250 dye. The protein bands were excised and in-gel digestion procedure was performed using classical protocol [8], without/with the addition of phenol, acetonitrile (ACN) or hydrogen peroxide solutions during in-gel enzymatic cleavage. ACN was added in concentrations 20 %, 40 %, 60 %, 80 %. Concentrations 2.5 mM, 5 mM, 10 mM, 20 mM for H₂O₂ and 12.5 M, 25 mM, 50 mM, 100 mM for phenol were tested.

2.3 Protein identification

Peptides obtained from in-gel or in-solution digestion were analyzed by MALDI-TOF/TOF 4700 Proteomic Analyzer (Applied Biosystems, Framingham, MA, USA). For the MS analysis, α-cyano-4-hydroxycinnamic acid was used as a MALDI matrix.

3 Results and Discussion

Hydrogen peroxide in aqueous solution with basic pH provides a hydroperoxide anion, which is a better nucleophile than a hydroxide anion, thus the addition of hydrogen peroxide might accelerate the process of enzymatic digestion. On the other hand, incorporation of more lipophilic phenol into the process of enzymatic hydrolysis with a formation of intermediary and easily cleavable phenyl esters could enhance the digestion as well. Water-miscible organic solvents may change the conformation of substrate and enzyme; increase the solubility of insoluble proteins without significant impact on activity of enzyme. In this way, they could increase accessibility of investigated proteins to trypsin, and so they could make enzymatic cleavage faster and more efficient. Therefore we decided to study and evaluate the effect of H₂O₂, phenol and ACN on the trypsin digestion and consequential MS identification of selected proteins representatives.

The most convenient experimental conditions were determined during optimization of digestion of bovine serum albumin (BSA), chicken ovalbumin and horse heart myoglobin. The use of optimal concentration of subsidiary agents and the duration of agent action were proved as the most important factors. Based on number

of matched peptides and protein sequence coverage (%), the concentration 2.5 mM H₂O₂ was determined as the most convenient for the analysis of our model proteins (as an example see Fig. 1). Similarly, 80% (v/v), and 50 mM solution were chosen as the most suitable for acetonitrile and phenol, respectively (data not shown here). The best results were obtained with the protocol where the agents were present only during 45 minutes of rehydration of gel particles with digestion buffer.

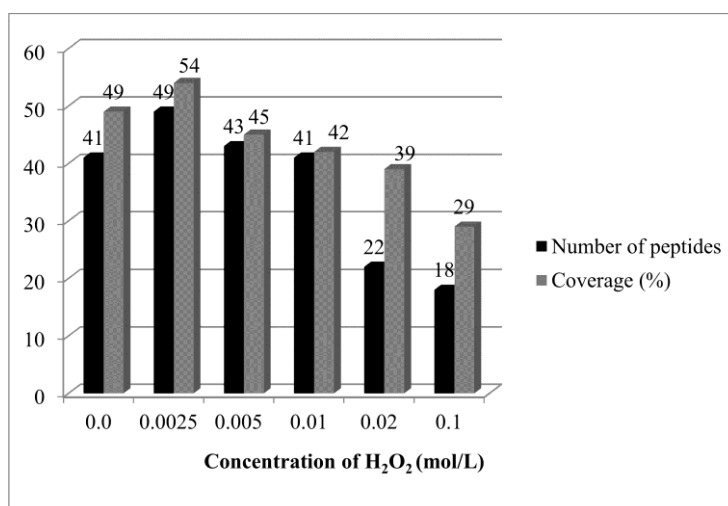


Fig. 1. Number of matched peptides and protein sequence coverage for BSA using different concentrations of added hydrogen peroxide.

Compared with the conventional method, addition of H₂O₂ solution enhanced the number of matched peptide fragments as well as sequence coverage and thus facilitate the MS identification of BSA (Fig. 2) and myoglobin (data not shown). On the other hand, analysis of ovalbumin, which is resistant to proteolysis, showed certain potential of ACN addition which slightly increase quantity of peptides in MS spectra (Fig. 2).

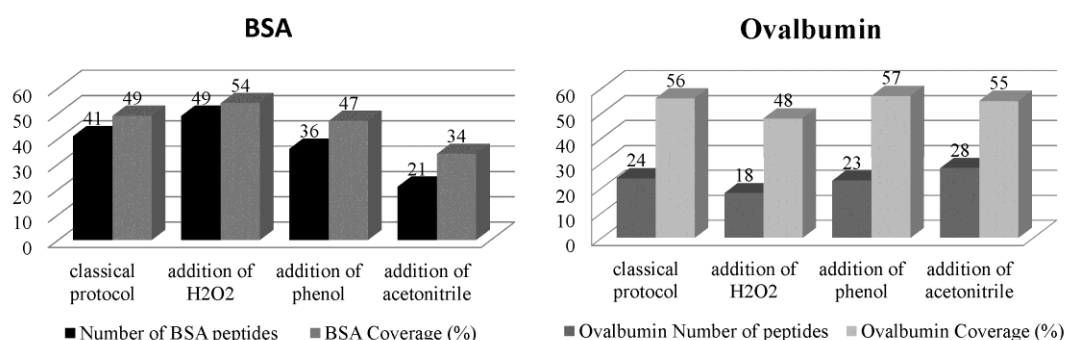


Fig. 2. Number of matched peptides and protein sequence coverage for BSA and ovalbumin by using different digestion methods (using the best experimental conditions for individual protocols).

4 Conclusions

MALDI-TOF MS results together with database searching indicated that the proteolysis in solvent system containing H₂O₂ and ACN has a potential for facilitation of identification of unknown proteins. Nevertheless, it is necessary to confirm these preliminary results by further experiments with more complex protein systems.

Acknowledgement

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P37 PARALLEL SINGLE-CELL ANALYSIS OF ACTIVE CASPASE-3/7 IN APOPTOTIC AND NON-APOPTOTIC CELLS

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Summary

Caspases are proteases that play key role in the process of apoptosis, the programmed cell death. Among them, caspase-3 and -7 are main executioner caspases that cleave many vital proteins during apoptosis and after their widespread activation, the process cannot be reversed. To analyze caspase-3/7 activation within single cells, a miniaturized device for parallel analysis of eight samples was developed. The assay is based on the modified luciferin-firefly luciferase bioluminescence (BL) system. Individual

suspended cells were collected and transferred into detection microvials using a micromanipulator. The bioluminescence was detected using a photon counting head with cooled photocathode. The LOD suitable for detection of active caspase-3/7 in both apoptotic and non-apoptotic cells was reached.

1 Introduction

Caspases are one of the many potential targets in cancer treatment and their activity is connected with many pathologies. Caspase-3 and -7 are particularly in focus because their activation during apoptosis marks the point of no return. Apart from their apoptotic functions, new information is emerging on their non-apoptotic functions as well. Increased activity of caspase-3 has been found for example during osteogenesis, brain development or skeletal muscle differentiation [1].

For the detection of active caspase-3/7, we used a commercially available Caspase-Glo[®] 3/7 reagent (Promega), a homogeneous BL assay based on modified luciferin-luciferase system developed by O'Brien et al. [2]. In this assay, aminoluciferin is conjugated with caspase-3/7 specific tetrapeptide Asp-Glu-Val-Asp which makes the substrate inaccessible for luciferase. After the peptide is cleaved off by the caspase, free aminoluciferin is immediately oxidized by firefly luciferase in presence of Mg²⁺, ATP and oxygen, resulting in a photon emission. After an initiatory period of time, the BL intensity reaches a steady state that lasts for tens of minutes and a flow of photons proportional to the concentration of active caspase-3/7 is generated. Due to the enzymatic nature of the assay, single caspase molecule generates multiple photons and the background signal is very low due to the absence of interfering autofluorescence, photobleaching and excitation light.

Here, we present a miniaturized device for parallel BL detection of active caspase-3/7 content in single apoptotic and non-apoptotic cells that is compatible with analyses of suspended cells selected and transferred by a micromanipulator mounted on a microscope. Our objective was to reach LOD below the average amount of active caspase-3/7 in a single non-apoptotic cell. To accomplish this, a sensitive assembly of photomultiplier tube (PMT) with a cooled photocathode working in the photon counting mode was implemented.

2 Experimental

Micromass cultures isolated from mouse forelimbs at embryonic day 12 were selected as a model. Two experimental groups were prepared: a control group cultivated in standard culture medium (non-apoptotic cells) and an experimental group treated with 5 μ M camptothecin for 8 hours (apoptotic cells). From one to six cells were collected at a time and transferred into individual detection microvials using ICSI micromanipulator (Eppendorf) with a 20 μ m o.d. holding pipette for *in vitro* fertilization, mounted on an inverted microscope Olympus IX71. The microvials were made from glass capillaries of sizes i.d./o.d. 1.3/1.9 mm by fusing at one end and each was filled with 4 μ l of Caspase-Glo[®] 3/7 reagent. After filling, all eight microvials were placed into a stainless steel revolving carousel vial holder and the whole assembly was mounted in front of the PMT detection window. The BL emission leaving individual

vials was detected consecutively and repeatedly in 5min intervals for a period of about 80 minutes until the steady state was reached in all vials. The detection assembly consisted of a photon counting head H7421-40 series with a heat sink and fan A7423, counting unit C8855-01 and power supply unit with temperature control M 9011 (Hamamatsu Photonics, Japan). The photon counting head is furnished with a PMT having a GaAsP/GaAs photocathode and a thermoelectric cooler. The cooler maintains PMT at a constant temperature of 0 °C and reduces thermal noise generated by the photocathode, thus increasing S/N ratio.

3 Results and Discussion

One of prerequisites of high sensitivity analysis of ultra-low volume samples is miniaturization resulting in reduction of background signal. The background signal of Caspase-Glo[®] 3/7 reagent linearly increases with its volume as the amount of accidentally released aminoluciferin accessible for luciferase oxidation rises. On the other hand, if the volume is too low, modified luciferin can be depleted before the BL reaches its steady state and the signal can be decreased. To reach the signal maximum, we used detection microvials of 1.3 mm i.d. filled with 4 µl of the reagent. This was enough to provide sufficient substrate delivery to the active caspase-3/7 molecules while maintaining relatively low background signal. Further decrease of the background was achieved by using a PMT with cooled photocathode. The photocathode yields a dark current of only about 3-6 photon counts/s which is very low compared to the blank signal of the reagent (about 62 counts per second).

The device was tested with apoptotic and non-apoptotic cells. In both cases, the caspase-3/7 content linearly increased with the number of cells. The average signal intensity for apoptotic cells ranged from 157-952 counts/s and was approximately three times higher than for the non-apoptotic cells where the signal ranged from 56-241 counts/s. High standard deviation were apparent particularly in the case of apoptotic cells. The deviations can be caused by cell heterogeneity, by the aspects of dynamics of camptothecin induced caspase activation in individual cells and the effect of the cell cycle phase of individual cells. It is also possible that camptothecin may not diffuse properly to the lower layers of cells in Petri dishes that adhere directly to the surface during cultivation. Some (very low) activation of caspase-3/7 in non-apoptotic cells can be due to the *ex vivo* approach and micromass treatment. It may also suggest some physiological basal level of activation or can even point to possible non-apoptotic functions of these caspases.

The LOD and LOQ were calculated as three and ten times the standard deviation of a blank, respectively. For apoptotic cells the LOD/LOQ were evaluated to be 0.27/0.86 of a caspase-3/7 content in an average apoptotic cell and for the non-apoptotic cells 0.46/2.92 of a caspase-3/7 content in an average non-apoptotic cell. The device is therefore suitable for both detection and quantification of active caspase-3/7 in individual apoptotic cells. In the case of non-apoptotic cells, caspase-3/7 can be reliably detected but not quantified.

4 Conclusions

We have developed a miniaturized device for sensitive detection of caspase-3/7 activity within single cells. The LOD reached is suitable for detection in both apoptotic and non-apoptotic cells but reliable quantification is so far possible only in apoptotic cells. Although the throughput of our device is limited, it could be in principle increased by using a microplate with 1536 wells each filled with 3–10 μ l of the reagent, providing a sensitive PMT detector monitoring each well separately without any crosstalk and fast and effective system for cell collection and transfer.

Acknowledgement

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P38 INVESTIGATION OF THE ADSORPTION AND DESORPTION OF SULFUR MUSTARD DEGRADATION PRODUCTS IN SEDIMENTS BY CAPILLARY ELECTROPHORESIS

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Summary

The aim of this work was to study the adsorption and desorption of sulfur mustard (HD) degradation products – thiodiglycol (TDG), thiodiglycol sulfoxide (TDGO) and thiodiglycol sulfone (TDGOO) – in sediments by using the capillary electrophoresis (CE) method with direct UV detection. The results of the study showed that the adsorption of TDG, TDGO, and TDGOO onto sediments was high and only a small quantity of the compounds could be desorbed. This investigation provides an explanation for why these main HD degradation products are so rarely found in the sediment samples taken from the dumpsites in the Baltic Sea.

1 Introduction

After World War II large amounts of chemical weapons were dumped in the sea. It is estimated that around 50,000 tons of chemical munitions was dumped in the Baltic Sea,

which contained approximately 15,000 tons of chemical warfare agents (CWAs) [1]. Sulfur mustard (HD), which is a blister agent, is the most abundant CWA dumped. HD accounts for two-thirds of the munitions dumped near Gotland and Bornholm [2]. In an aqueous environment, HD degrades predominantly by hydrolysis to TDG. TDG may be oxidized to TDGO and, more slowly, to TDGOO (Fig. 1.) [3, 4]. The analysis of HD degradation products in sediments indicates the presence and leakage of HD from the corroded shells. This information, in turn, is important to evaluate the environmental hazards caused by munitions dumping. Surprisingly, HD and its degradation products TDG, TDGO and TDGOO are rarely found in the sediment and water samples taken from the dumpsites [5, 6]. This is very astounding considering that over 60% of all dumped CWAs include mustard gas [5]. The purpose of this study was to investigate the adsorption and desorption of TDG, TDGO and TDGOO onto and from sediments to understand and explain why these HD degradation products are found in so few samples taken from the dumpsites.

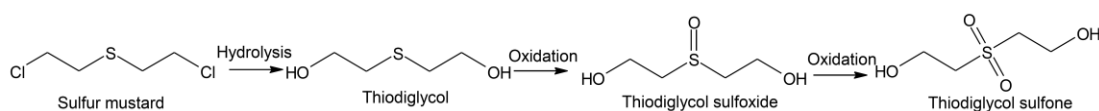


Fig. 1. Hydrolysis and oxidation of sulfur mustard.

2 Experimental

The CE method with direct UV detection was applied for the determination of TDG, TDGO and TDGOO in sediment samples [7]. Sediment samples were collected at the Port of Virtsu (the Baltic Sea, Estonia). It was verified that the samples did not contain any degradation products of HD. For sample preparation, 1 g of dry sediment was spiked with a 50, 150, 200, 250 or 500 μM standard solution of three HD degradation products. The added amount of solution was 2.5 mL. After a 24 h storage in the refrigerator (+4 $^{\circ}\text{C}$) water was removed by centrifugation (20 min, 10 000 rpm). The collected water was extracted using powdered carbon aerogels to determine the content of TDG, TDGO and TDGOO. Acetonitrile (ACN) was used for the elution of compounds of interest from carbon aerogels. The remaining dry sediment was extracted with ACN. Both ACN fractions (from carbon aerogels and dry sediment) were evaporated to dryness and subjected to the derivatization and analysis procedure [7].

3 Results and Discussion

The adsorption and desorption of HD degradation products in sediments after a 24 h storage were investigated. With no adsorption at all the concentration of TDG, TDGO and TDGOO in collected water would have been 50, 150, 200, 250, and 500 μM . In reality, all these concentrations were lower as some amount of HD degradation products was adsorbed onto the sediment. The differences between these two concentrations were well suited for estimating the degree of adsorption of HD degradation products in sediment samples. The dry sediment (water had been removed by centrifugation) was extracted with ACN to estimate the extent of desorption. With concentrations of 50 and

150 μM , most of the TDG and TDGO had adsorbed onto the sediment ($> 85\%$). At the same time, only a small amount (not more than 15% from the initial concentration) of HD degradation products could be desorbed (tried with ACN and dichloromethane) from the sediment. The high rate of adsorption of TDG, TDGO and TDGOO onto sediments and the low rate of desorption from sediments might explain why these compounds were found in so few samples taken from the HD dumpsites. It can be seen from Figure 2 that the adsorption of TDG onto the sediment is the highest and that of TDGOO, the lowest. As expected, the desorption of the HD degradation products was the other way around: the desorption of TDGOO from the sediment was the highest and that of TDG desorption, the lowest.

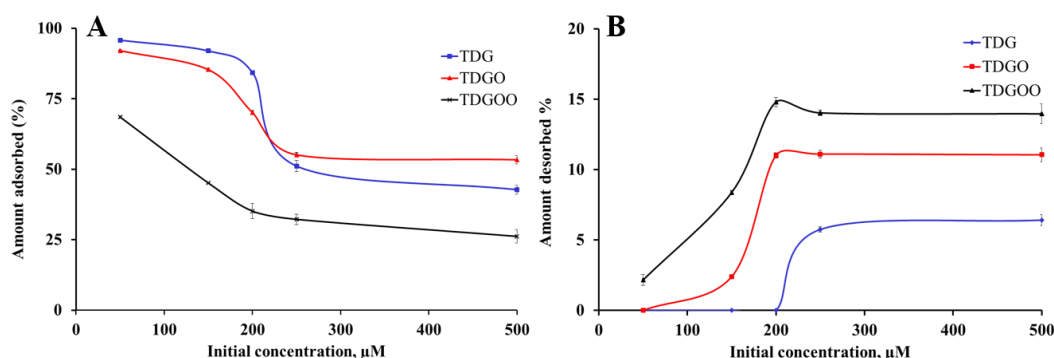


Fig. 2. Adsorption (A) and desorption (B) of TDG, TDGO, and TDGOO onto and from sediments after a 24 h storage.

Additionally, it was noticed that after the storage of the spiked sediment (spiked only with TDG) in the refrigerator ($+4\text{ }^{\circ}\text{C}$) for 24 hours, 15% of TDG was oxidized to TDGO. TDGO did not oxidize to TDGOO within 24 hours. It was also established that after the storage of the spiked sediment in the refrigerator for 7 days, no TDG was detected, and the concentrations of TDGO and TDGOO had decreased six and two times, respectively (Fig. 3).

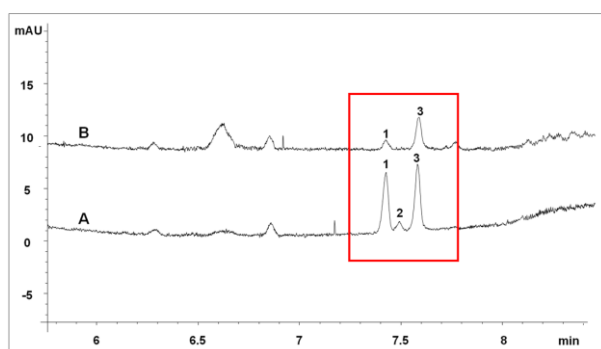


Fig. 3. Electropherograms of desorption of compounds. A – TDGO (1), TDG (2) and TDGOO (3) after storing the spiked sediment in the refrigerator for 24 hours, B – TDGO and TDGOO after storing the spiked sediment in the refrigerator for 7 days.

4 Conclusions

The adsorption and desorption of HD degradation products onto and from sediments were studied. The results demonstrated that the adsorption of TDG onto the sediment was the highest and that of TDGOO, the lowest. At the same time, desorption of TDGOO from the sediment was the highest and that of TDG, the lowest. Overall, the adsorption of TDG, TDGO, and TDGOO onto sediments was high and only a small quantity of the compounds could be desorbed. The results of this work might explain why the HD degradation products TDG, TDGO, and TDGOO are rarely found in the sediment and water samples from the dumpsites. Nevertheless, further investigations are needed.

Acknowledgement

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Summary

Matrix-assisted laser desorption/ionization mass spectrometry was introduced for the analysis of enzyme kinetics and inhibition. The K_m for selected substrate and IC_{50} values for enzyme inhibitor were in a good agreement with results obtained by capillary electrophoresis coupled to electrospray mass spectrometry. More specifically, it was focused on β -secretase (BACE), an enzyme playing important role in Alzheimer's disease development. Its selective inhibition in brain could be beneficial for the patients; thus, specific inhibitor of this enzyme was examined as well. In comparison with traditional fluorometric or colorimetric methods, the both mass spectrometric approaches offered faster, sensitive and more precise BACE analysis.

1 Introduction

Alzheimer's disease (AD) is a serious neurodegenerative illness, affecting around 44 million people worldwide. It is characterized by accumulation of amyloid plaques and neurofibrillary tangles in brain, leading to the symptoms such as memory loss and inability to perform simple daily tasks [1].

The β -site APP-cleaving enzyme (BACE, β -secretase) is supposed to play the crucial role in the AD development. This enzyme from the family of aspartic proteases cleaves transmembrane APP protein on β -site and is followed by γ -secretase cleavage on γ -site. BACE overexpression leads to the production of $\alpha\beta$ peptide ($\alpha\beta_{42}$ rather than $\alpha\beta_{40}$ isoform) and to the development of toxic amyloid plaques in brain. Although the biochemistry of AD is still not fully understood, the inhibition of BACE thought to help in the prevention of AD progression and thus various inhibitors are searched for nowadays [2].

BACE activity for selected substrate can be measured in many ways. There is a commercial fluorescence resonance energy transfer (FRET) assay with fluorescently marked substrates based on the enzymatic cleavage and strong, real-time fluorescent signal production. However, this approach is relatively insensitive and requires high concentrations of the enzyme. An improved biochemical assay suggests fluorescent/colorimetric detection of more suitable substrates and provides increased sensitivity [3]. Another option is the detection of products after the reaction of the enzyme and unlabelled substrate, e.g. by mass spectrometry (MS). Two distinctive techniques were established for this purpose, the first involving the separation of particular species by capillary electrophoresis (CE) and their electrospray mass

spectrometry (ESI MS) detection. The advantages of this method are both sensitivity and preciseness, yet, it includes the time consuming separation step [4].

The separation may be completely omitted if using another MS technique, matrix-assisted laser desorption/ionization (MALDI), which employs a UV-laser to desorb and generate molecular ions from the sample surface [5, 6]. Although MALDI is highly resistant to sample contamination and it can produce charged molecules directly from unprocessed biological samples, great amount of salts may suppress analyte signals. This is also the case of samples in our enzymatic study, where the buffer environment with naturally high content of salts is used. Therefore, standard ZipTip purification is necessary. While MALDI MS is not perceived as a quantitative method in general, we showed that with a suitable internal standard, it can become an easy and accurate method useful in enzymatic research.

2 Experimental

2.1 Biochemical sample preparation

Recombinant human β -secretase (BACE), its inhibitor and internal standard for CE-MS were purchased from Sigma Aldrich. The substrate [Asn⁶⁷⁰, Leu⁶⁷¹]-APP fragment 667-676, proteolytic DAEFR product and internal standard for MALDI were purchased from Bachem.

Three types of samples were prepared – kinetic, inhibitory and model. The model samples contained the DAEFR product in the concentration range 2-40 μ M and similar reactants as kinetics samples; however the enzymatic reaction was not started.

The kinetic samples consisted of the BACE enzyme and its substrate in the concentration range 100-1000 μ M. The mixture was incubated for one hour in sodium acetate buffer pH 4.25, leading to DAEFR production. The enzymatic activity was stopped by freezing the samples at -80 °C. The inhibitory samples were prepared similarly to the kinetic samples with the addition of the inhibitor.

2.2 MALDI sample preparation

First, a suitable internal standard had to be selected. In the original CE-ESI MS method, angiotensin II was found as an appropriate internal standard; however, it could not be used in the case of MALDI MS. Therefore, the DGEFR standard, differing only in a single amino acid from the product, was proposed. After the IS addition, samples were desalted by ZipTip C18 stationary phase tips. Sample eluates were pipetted on a MALDI metal target and mixed with CHCA matrix by quick and dirty technique. Four to six spots were generated for each sample.

2.3 Measurements and data processing

Samples were measured on the MALDI-TOF mass spectrometer (Autoflex speed, Bruker). Each spot was exposed to several thousand shots of randomly located laser (70-80 μ m in diameter) in the reflector mode, with the detection window 580-760 Da and matrix signal suppression. The intensities of DAEFR and DGEFR peaks were calculated by FlexAnalysis software and final kinetic and inhibition results were evaluated in MS Excel and SigmaPlot software.

3 Results and Discussion

3.1 Quantitative potential of MALDI

First, angiotensin II was tested as the internal standard for MALDI. However, it was found inappropriate as its response anti-correlated with that of DAEFR. In contrary, the DGEFR standard proved to be the suitable IS which allowed accurate DAEFR quantification in the model samples.

3.2 Enzyme kinetics and inhibition

The kinetics of BACE enzyme for selected substrate was determined from DAEFR content at various initial substrate concentrations. Acceptable sigmoidal curves and K_m values for the selected substrate levels were obtained. Freezing was found not to stop the enzymatic reaction completely, resulting in a certain spread of multiplicates. In inhibition studies, a selected BACE inhibitor was examined and its IC_{50} was calculated.

4 Conclusions

In this work, MALDI-TOF MS was applied for the analysis of BACE kinetics and inhibition. Compared to the fluorescent and colorimetric assays, sample preparation for MALDI MS is simple and the method provides precise results which are comparable with those of the CE-ESI MS approach.

Acknowledgment

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**P40 MORPHOLOGY AND PHOTOSYNTHESIS BASED SCREEN
TO UNRAVEL MECHANISM OF ROS-AUXIN CROSSTALK**

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Summary

Plants are continuously exposed to a variety of external conditions able to affect their growth, development and productivity. Reactive oxygen species (ROS) are produced as a normal product of plant cellular metabolism. As various environmental stresses lead to excessive production of ROS causing progressive oxidative damage and ultimately cell death, plants have developed a complex redox signaling network that exerts a high degree of control over ROS accumulation. Responses to ever-changing environment are also mediated by plant hormones. Recent evidences indicate that among phytohormones, auxins play crucial role in response of plant to environmental cues. Moreover, auxin pathway seems to be involved in the modulation of photosynthesis upon stress [1].

Although in most of the studies ROS-auxin crosstalk have been limited to a mere observation, stress responses in plants undoubtedly are tightly coordinated with developmental processes. To uncover how ROS-auxin interplay integrates stress signals and plant development and how the reciprocal interplay between photosynthetic and auxin signals modulate stress induced morphological response (SIMR), we are performing morphological and Photosystem II chlorophyll fluorescence based screening. The combination of both screenings results in 3 different groups of auxin-related lines: lines with only altered SIMR, lines with only altered photosynthesis, and lines with both SIMR and photosynthetic effects.

Here we show an example of the utility of the screening for the selection of auxin-related genes involved in SIMR and particularly acting in auxin transport from the PIN-FORMED (PIN) auxin efflux carrier family. Expression analysis of different promoter-GUS fusion lines grown under stress conditions reveals that PINs gene expression is differentially regulated by different stressors. Moreover, altered PIN6 levels in Arabidopsis plants affect stress-induced growth retardation and photosynthesis responses.

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- 3 - EMMAG, Exchange of Excellence between Europe, Maghreb and Egypt.

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P41 SENSITIVE DETERMINATION OF SEVNL AND DAEFR PENTAPEPTIDES AS BETA-SECRETASE PRODUCTS BY CE-C⁴D FOR ITS ACTIVITY ASSAY

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Summary

Alzheimer's disease is the most common form of dementia; however, effective treatment is not yet available. Mechanism of the disease involves formation of neurotoxic oligomers of β -amyloid peptides. These peptides are produced mainly by β -secretase thus inhibition of the enzyme might be a way to treat the disease. In order to study enzyme's inhibition – preferably with unlabeled substrates – high method's sensitivity is demanded. Consequently, highly sensitive capillary electrophoresis based method with contactless conductivity detection was developed.

1 Introduction

Despite intensive research and ongoing clinical trials, effective causal or symptomatic treatment of Alzheimer's disease is not yet available. The disease is manifested with decline in memory and cognitive functions leading to progressive disorientation in time and place, and eventually death. The brains of the patients are histopathologically characterized by two hallmark lesions: i) cerebral plaques laden with β -amyloid ($A\beta$) peptide and ii) neurofibrillary tangles. Two proteins are responsible for progression of the disease owing to their neural and mitochondrial toxicity: the hyperphosphorylated tau protein and soluble oligomers of $A\beta$ peptides, mostly of 42 amino acids in length. $A\beta$ peptides originate from proteolysis of amyloid precursor protein by the sequential enzymatic action of beta-site amyloid precursor protein-cleaving enzyme 1 (BACE-1 or β -secretase) and other enzymes. Therefore the inhibition of these enzymes could be a strategy to treat the disease [1, 2]. The β -secretase inhibition studies are conducted mainly using high-throughput Fluorescence Resonance Energy Transfer (FRET)-based assays; however, fluorescence quenching by tested compounds has been reported thus the assay's accuracy is limited [3]. Separation-based methods might overcome this issue and by use of principally different detection technique, enzyme assay can be conducted with unlabelled substrates. For such purpose, capillary electrophoresis coupled with – in general simple and inexpensive – contactless conductivity detector (CE-C⁴D) was successfully applied.

2 Experimental

2.1 CE-C⁴D method

Agilent 7100 CE System (Agilent Technologies, Waldbroon, Germany) was used in all experiments. The C⁴D was custom-made with working frequency 1 MHz which has been described in detail elsewhere [4]. Background electrolyte (BGE) was comprised of 10 mM pyridine-2-carboxylic acid titrated with imidazole to pH 5.45. Hydrophilic polymer poly(vinylpyrrolidone) was added to BGE at concentration 1 % (w/w) to reduce electro-osmotic flow. The separation voltage was -30 kV, the cassette temperature 25 °C and the sample was injected by pressure 100 mbar for 30 s. The bare fused-silica capillary of 50/375 µm (id/od) and of 48.0/34.0 cm total/effective lengths was used in all experiments.

2.2 Transient isotachopheresis

In order to improve peak shape and increase sensitivity, transient isotachopheresis (tITP) was induced by immersing the inlet capillary end into the buffer comprised of 10 mM MES/NaOH, pH 5.60, and the voltage defined by the constant current 2 µA was applied for 60 s. MES anion acts as terminal electrolyte, whereas acetate (ca. 5 mM) from the sample acts as leading electrolyte.

2.3 Enzyme assay

Standard “Swedish mutation” substrate for β-secretase of single letter amino acid sequence SEVNLDAEFR was used in kinetic and inhibition studies. The β-secretase cleaves SEVNLDAEFR to pentapeptides SEVNL and DAEFR. Enzyme assay was conducted in 50 mM sodium acetate/acetic acid, pH 4.25 with 20 U/ml of β-secretase and 0.1-1 mM SEVNLDAEFR (or 0.2 mM SEVNLDAEFR and inhibitor). The reaction was incubated at 37 °C and 500 rpm for an hour using Eppendorf Thermomixer comfort (Eppendorf, Hamburg, Germany). The reaction was quenched by the addition of acetonitrile of volume 1.5× the volume of the reaction mixture.

3 Results and Discussion

3.1 Optimization of BGE

Suitable pH of BGE was selected on the basis of theoretical dissociation of the peptides SEVNLDAEFR, SEVNL and DAEFR resulting in charge differences. Dissociation curves are depicted in Fig. 1 and were constructed using pK_a's of free amino acids and ProMOST approach [5]. The first approach showed better agreement with experimental data and implies good resolution around pH 5. At this pH, all peptides are negatively charged, thus being detected as anions.

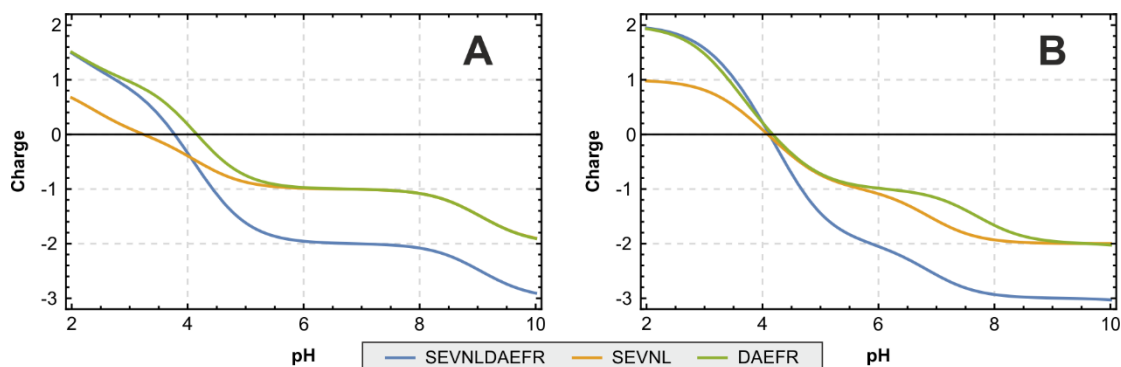


Fig. 1. Predicted charge in dependence on pH of peptides SEVNLDAEFR, SEVNL and DAEFR calculated using pK_a 's of free amino acids (A) and using ProMoST approach [5] (B).

Optimization of the acidic component of BGE were conducted using 10 mM acetic acid (pK_a 4.76), MES (pK_a 6.1) or pyridine-2-carboxylic acid (2Pic; pK_a 5.39) titrated with 1 M 2,6-dimethylpyridine to pH 5.60. 2Pic based BGE provided the best signal-to-noise (S/N) ratio among the other tested BGEs, thus was used for further optimization. Basic component of BGE can affect S/N [6], therefore several BGEs comprised of 10 mM 2Pic titrated with 1 M pyridine (pK_a 5.23), 3-methylpyridine (pK_a 5.70), 2,6-dimethylpyridine (pK_a 6.65), L-histidine (pK_a 6.04), imidazole (pK_a 6.99), *N*-ethylmorpholine (pK_a 7.67), piperazine (pK_{a1} 9.73; pK_{a2} 5.33) or NaOH to pH 5.60 were prepared. In case of 2Pic/piperazine BGE, substantial wavy-like noise was observed most likely due the high conductivity of piperazine cations. Best S/N was recorded for BGE comprised of 2Pic/imidazole thus being considered optimal.

3.2 Optimization of transient isotachopheresis

Optimization of BGE was conducted on model sample; however, severe defocusing of the peaks was observed when the model sample was fortified with 0.1 M acetic acid/NaOH buffer (pH 5.60). The cause for defocusing could be in the mobility of the peptides being outside the isotachophoretic region, i.e. between mobility of acetate (leading electrolyte) and 2Pic from BGE (terminal electrolyte). Therefore, inlet capillary end was immersed into 10 mM MES/NaOH, pH 5.60 after the sample injection to extend the isotachophoretic region covering the mobility of the peptides. Optimal time of tITP step depends on sample composition, injected plug length and applied current. As a result, optimization of this parameter was done on pseudo-real sample where β -secretase was spared and its activity was simulated by addition of SEVNL and DAEFR standards in equimolar concentrations (substrate-product ratio, 20:1). Acetate concentration was kept constant at 5 mM, tITP was done at constant current $-2 \mu\text{A}$ and the sample was injected by pressure 100 mbar for 30 s. Peak heights increased up to 45 s of tITP, above 90 s impurities from BGE being focused and reaching the region in electropherogram where analytes migrates. The optimal time of tITP was set 60 s as it provides repeatable focusing of the sample having minor changes in composition (e.g. enzyme-related changes) and the analyte's peak are

recorder over clear baseline. The limits of detection ($S/N=3$) for SEVNL and DAEFR under optimal conditions were 20 nM and 25 nM, respectively. Inter-day peak area precision ($n=12$, 3 days) were 3.1 % and 2.9 % for SEVNL and DAEFR, respectively. As a result both products can be used for the β -secretase activity evaluation with close analytical parameters.

4 Conclusions

The presented CE-C⁴D method was developed with respect to the high sensitivity in order to study inhibition of β -secretase which might be possible treatment of Alzheimer's disease. The method's sensitivity was assured by thorough BGE optimization and preconcentration using tITP. C⁴D in principle offers detection of peptides in their native form thus the CE-C⁴D can be used to confirm results obtained by mass-spectrometry.

Acknowledgement

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P42 HIGHLY SENSITIVE ANALYSIS OF CHLOROPHENOLS AND SULFONAMIDES IN WATERS BY ELECTROPHORETIC FOCUSING ON INVERSE ELECTROMIGRATION DISPERSION GRADIENT WITH ESI-MS DETECTION

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Summary

This work presents a new methodology for high-sensitivity analyses by capillary electrophoresis (CE) with electrospray-ionization mass spectrometric (ESI-MS)

detection, based on electrophoretic focusing on an inverse electromigration dispersion (EMD) gradient. The technique is based on a robust electrolyte system providing a gradient of required properties, suitable for routine analyses of trace amounts of weak acids with pK_a values between approx. 6.5 and 9. Examples analyses of several chlorophenols and sulfonamides are presented, with LODs around 3×10^{-9} M for spiked drinking water without any sample pretreatment.

1 Introduction

In 2010, we have described a new electrophoretic separation principle, focusing of nonamphoteric weak ionogenic analytes on inverse electromigration dispersion profiles [1]. Here we present its first practical implementation, namely on a CE system with ESI-MS detection.

2 Experimental

We used an Agilent 7100 CE system with a 100 μ m id bare fused-silica capillary, CE-ESI-MS interface and 6130 single quadrupole mass spectrometer. The leading electrolyte was 15.2 mM maleic acid with 21.7 mM 2,6-lutidine at pH 5.81, the terminating electrolyte was 2 mM maleic acid and 27.6 mM 2,6-lutidine at pH 7.53. Electrophoresis was performed in reversed polarity mode at 20 kV.

3 Results and Discussion

The electrolyte system with maleic acid and lutidine not only provides the gradient of required properties but also sufficient ESI-MS compatibility (volatility). The proposed methodology ensures that the analytes are focused a particular position of the migrating gradient, concentrated, separated, transported to the detector by superimposed electromigration, electroosmotic flow and ESI suction, and selectively detected by the MS detector. Fig. 1 presents an example of a very sensitive analysis of direct injection (without any sample pretreatment) of drinking water spiked with $5 \cdot 10^{-9}$ M of three chlorophenols, with baseline separation in 15 minutes.

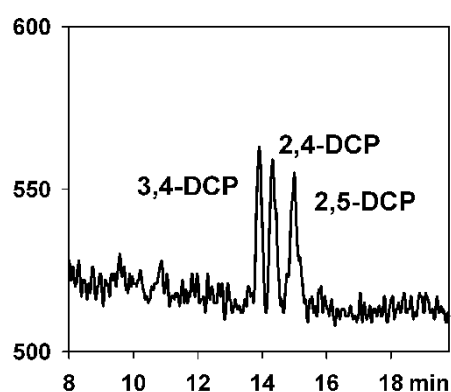


Fig. 1. Analysis of 2,4-dichlorophenol (2,4-DCP), 2,5-dichlorophenol (2,5-DCP) and 3,4-dichlorophenol (3,4-DCP). The sample was drinking water spiked with $5 \cdot 10^{-9}$ M of the three analytes. Injection 100 mbar/100 s, voltage 20 kV, fragmentor 130 V, negative SIM data monitored for m/z 161 and 163.

4 Conclusion

The applicability of focusing on inverse EMD profiles in analytical practice is demonstrated by successful combination with ESI-MS detection. The designed electrolyte system is suitable for separation and analysis of weak acids of pKa values between approx. 6.5 and 9.0. The presented analyses of chlorophenols and sulfonamides in waters with LODs around $3 \cdot 10^{-9}$ M demonstrate the potential of this methodology for fast and high sensitivity analyses of untreated samples.

Acknowledgement

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P43 INVESTIGATION OF SURFACE CHARACTERISTICS OF MICROFLUIDIC SUBSTRATES (PDMS) BY INVERSE GAS CHROMATOGRAPHY

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Summary

Microfluidic devices become more and more important in the analytical and life science fields. This technology, also known as lab on a chip, makes analytical measurements easier, faster and amenable in HT environment. One of the main advantages of this technology is its size. However, its usage is limited by the substrates it is made of. Its surface properties often improved by specific treatments, however, it is challenging to accurately determine the effect of these treatments. iGC (inverse gas chromatography) was proved to be a reliable, accurate method to characterize the surface of treated and untreated PDMS substrates.

1 Introduction

Poly(dimethylsiloxane) (PDMS) is a frequently used material for microfluidic substrates due to its favorable properties, such as easy fabrication for rapid prototyping, reproducibility down to sub-micron levels, biocompatibility, chemical inertness, optical

transparency, gas permeability and electrical resistivity just to list the most important ones. However, it has drawbacks like hydrophobicity with concomitant non-specific binding of hydrophobic biomolecules. Several methods have been developed to eliminate these disadvantages, for example chemical modification, surface coverage and oxygen plasma treatment. This latter one is one of the most commonly used, in spite of the fact that it provides only a temporary remedy, lasting for a couple of hours before the original surface is recovered. Therefore, information about the time requirement of this surface recovery process is essential for planning the appropriate experimental steps.

2 Experimental

A two-component silicon elastomeric polydimethylsiloxane (PDMS) kit (SYLGARD 184) from Dow Corning (Midland, MI, USA) was used as base material of the samples. The fabricated PDMS was cryogenically grinded at liquid nitrogen temperature (-196°C) by a CryoMill (Retsch, Haan, Germany). The surfaces of cryomilled sample particles were treated by synthetic-air plasma from 2-10 s at a plasma power of 500 W using a home-made microwave oxygen plasma reactor under vacuum. The efficiency of the different treatment times was investigated by a second generation inverse gas chromatograph, by the Surface Energy Analyzer (IGC-SEA) of Surface Measurement Systems Ltd. (London, UK).

3 Results and Discussion

Measurements of the surface energy of solid materials provided information about the actual characteristics of the surface. The dispersive component of surface energy was determined by the Dorris-Gray method describing the van der Waals interactions, while the specific component of surface energy expressed the surface ability for Lewis acid-base interactions. The two components of surface energy were determined for treated and untreated PDMS samples by iGC. The results showed the optimal oxygen plasma treatment time of PDMS and iGC proved to be an excellent method to investigate the recovery process of the treated surfaces. IGC is proved to be an accurate method to determine the effect of the plasma treatment on the surface of PDMS. IGC-SEA is a unique apparatus to accurately characterize PDMS substrates based on quantitative energetic analysis of the surfaces to better understand their adhesion, cohesion and adsorption features. The oxygen plasma treatment activates the surface by oxidation and radical substitution the methyl groups to hydroxyl, hydroxymethyl and carboxyl functional groups. However, these obtained functional groups are ephemeral, continuously transform back to the original inactive surface having methyl groups. The IGC experimental results indicated that the etched surface was transient: the PDMS recovered within several hours after plasma treatment.

Acknowledgement

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**P44 CAPILLARY ELECTROPHORESIS-FRONTAL ANALYSIS
FOR THE CHARACTERIZATION OF DRUG-METAL BINDING**

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Summary

This study examined the possibilities of using capillary electrophoresis-frontal analysis (CE-FA) for the characterization of drug-metal binding parameters such as formation constant (K_f) and number of binding sites. Deferiprone and iron in the Fe(III) form were selected as a model system. Apparent K_f value was obtained using nonlinear regression. The results provide a more quantitative picture of how this drug binds with metal and illustrate how CE-FA can be used to examine drug-metal interactions.

1 Introduction

The formation constant (K_f) is commonly used to describe the strength of binding between chelator and ligand. In our case, the chelator is a commonly used iron chelator drug called deferiprone (CP20) and the ligand is iron in the Fe(III) form. The strength of the interaction plays an important role in different application areas including chemistry, biology and medicine. CP20 is typically prescribed for the treatment of β -thalassemia to help lowering iron levels in the blood. Currently, the drug plays a role in the treatment of other diseases such as cancer, renal insufficiency and HIV. Therefore, the investigation of its binding mode under different conditions is of interest [1-3].

The use of CE-FA for metal complex binding studies has been reported by Erim et al. [4] and it seems to be relevant tools for such characterization. The advantages of capillary electrophoresis (CE) as a separation method for the determination of K_f are very low sample consumption, high resolution and unnecessary of using highly purified samples. Additionally, immobilization or labeling are not required. Furthermore interactions in CE take place in solution, which can better simulate native conditions [4, 5].

The main objectives of this study were to determine the binding parameters as K_f and stoichiometry of CP20 with its typical ligand – Fe(III) using CE-FA.

2 Experimental

2.1 Chemicals

All reagents were obtained in analytical grade. Sodium hydroxide, sodium tetraborate decahydrate, ferric chloride hexahydrate and EDTA were obtained from Sigma-Aldrich (Steinheim, German), hydrochloric acid was obtained from Fluka (Buchs, Switzerland).

The BGE in all experiments was the 20 mM borate buffer, pH 10.5. The samples were prepared by dissolution in this buffer. All solutions were prepared using deionized water from a Millipore system (Merck, Milford, MA, USA).

2.2 Instrumentation

All experiments were performed in an Agilent ^{3D}Capillary Electrophoresis System (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array UV-Vis detection system. The analyses were carried out in a 75 μm I.D., 375 μm O.D. uncoated fused silica capillary with length 48.5/8.5 cm ($L_{\text{tot}}/L_{\text{eff}}$) from Polymicro Technologies (Phoenix, AZ, USA) thermostated at 25 $^{\circ}\text{C}$. The samples were injected into the capillary with a pressure of 10 mbar for 50 s at the cathode end of the capillary. Operational voltage of 10 kV was applied in reversed polarity and the detection wavelength was set to 280 nm (CP20) and 450 nm ($[\text{Fe}(\text{CP20})_3]$ complex).

3 Results and Discussion

3.1 Optimization, validation of method and determination of formation constants

Firstly, CE-FA method had to be optimized for selected system. The time and sufficient repeatability of the analysis were important requirement of this method. Therefore, the following parameters have been optimized: the capillary length, separation voltage, injection time and flushing procedure between runs.

The run-to-run repeatability of analysis, linearity in concentration range, detection and quantitation limits were evaluated for CP20 after performed optimization and the method was finally used to determine K_f of $[\text{Fe}(\text{CP20})_3]$ complex whose chemical structure is shown in Fig. 1. CE-FA provides good repeatability and results of K_f and stoichiometry agree with literature.

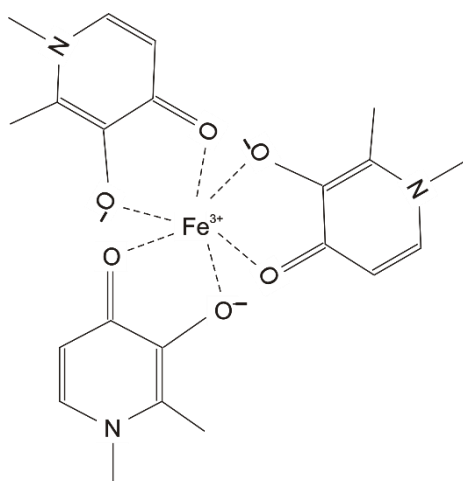


Fig. 1. Chemical structures of $[\text{Fe}(\text{CP20})_3]$ complex.

4 Conclusions

The main aim of this work was to characterize the affinity binding and stoichiometry between CP20 and iron in the Fe(III) form.

The method for determination of K_f and stoichiometry of model system was developed. Apparent K_f value obtained using nonlinear regression was in agreement with value published in literature [3].

Acknowledgement

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P45 MONITORING OF QUANTUM DOTS FORMATION INDUCED BY UV LIGHT

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Summary

In this paper, we focus on systematic study of low temperature, and fast synthesis of water soluble QDs stabilized by mercaptosuccinic acid (MSA) by UV irradiation ($\lambda = 245$ nm). The effect of UV irradiation on the QDs have been investigated. The best results were achieved with a mixture of precursors containing cadmium, selenium and MSA. The main contribution of this work is the use of UV light ($\lambda = 245$ nm) for the direct synthesis of CdSe QDs (quantum yield 13.4%) by UV transilluminator.

1 Introduction

Luminescent nanoparticles with a size range of units or tens of nanometers, known as quantum dots (QDs), are nowadays favored due to their unique optical as well as electrochemical properties. Wide spectrum of applications caused enormous interest in their synthesis and modification. Top-down (physical) methods include different types of lithographic techniques (laser, ion and X-ray) or etching and grinding. However, more popular is methods the bottom-up (chemical) approach. The bottom-up processes include a variety of methods such as microwave-, ultrasonic- or photochemicals-assisted technique, high temperature technique and biosynthesis [1, 2]. Another option is the interaction with UV light demonstrated in this work.

2 Experimental

2.1 Preparation of QDs precursors

10 mL of cadmium(II) acetate ($5.3 \text{ mg}\cdot\text{mL}^{-1}$) and 1 mL of mercaptosuccinic acid solution ($60.0 \text{ mg}\cdot\text{mL}^{-1}$) were mixed with 76 mL of deionized water on a magnetic stirrer. Then 1.5 mL of sodium selenite ($5.3 \text{ mg}\cdot\text{mL}^{-1}$) was also added under continuous stirring.

2.2 Formation of quantum dots by UV light irradiation

3 mL of precursor solution was irradiated by $\lambda_{\text{em}} = 254 \text{ nm}$. Energy of light applied to the sample was determined as $E = 0.14 \text{ mW}/\text{mm}^2$.

3 Results and Discussion

3.1 Formation of CdSe-QDs from precursors monitored by spectroscopic techniques

It is shown that increasing the illumination time caused an increase in the intensity and a shift of the absorption maximum (Fig. 1 A), also an increase in the emission intensity was observed (Fig. 1B) during first 30 minutes of illumination, however in the time interval 35-60 minutes of illumination, the fluorescence intensity decreased. After 5 minutes of illumination, the emission maximum was $\lambda_{\text{em max}} = 420 \text{ nm}$, but after 60 minutes, the maximum increased for 158 nm to 578 nm. The presented results confirm that the UV illumination causes formation of the fluorescent nanocrystals stabilized by the MSA via the thiol group, demonstrated by the red shift of the absorption and emission spectra.

Using the dynamic light scattering technique the increasing particle size dependent on the time of illumination was confirmed. It was observed that after 5-minute illumination, QDs with the size of $2.3 (\pm 0.4) \text{ nm}$ and zeta potential of $-48 (\pm 6) \text{ mV}$ were formed and after 15-minute illumination, the size and increased to $3.1 (\pm 0.3) \text{ nm}$. Finally, the illumination for 30 minutes caused the formation of nanoparticles with the size of $4.1 (\pm 0.4) \text{ nm}$ and zeta potential of $-45 (\pm 5) \text{ mV}$. The obtained results suggest that the solution resulting by the UV light illumination was stable, because the solution with zeta potential of absolute value above 30 mV is generally considered non-aggregating and stable [3]. The photographs of the QD solutions in UV light are shown in Fig. 1C.

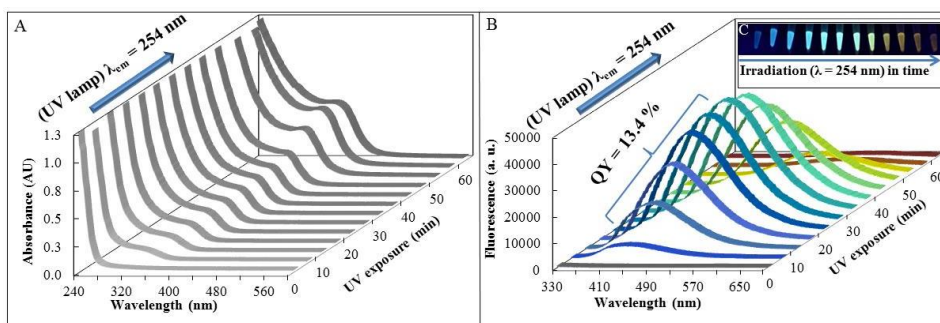


Fig. 1. Spectral analysis of UV-formed QDs, A) Absorption spectra of CdSe QDs (240 - 600 nm) obtained after illumination of precursors (0 – 60 min) by UV light, B) Fluorescence emission spectra (330 – 700 nm) dependent on illumination time (0 – 60 min), C) Photographs of the UV-formed QDs under UV illumination.

4 Conclusions

Based on the best fluorescent properties, the precursors composed of Cd and Se (without reduction agent) were selected for further testing due to the highest QY (13.4%). Subsequently, the formation of QDs was monitored by spectroscopic techniques. The biggest advantage of UV formation of QDs is in the possibility of synthesis of QDs in very small volumes.

Acknowledgement

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**P46 STUDYING OF COMPETITIVE INTERACTIONS
OF DRUGS WITH HUMAN SERUM ALBUMIN BY CAPILLARY
ELECTROPHORESIS-FRONTAL ANALYSIS**

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Summary

Drug-protein interactions have a significant effect on pharmacodynamics and pharmacokinetics properties of drugs. Bound fraction of drugs is reservoir, whereas free fraction provides pharmacological effect. When more drugs are administered simultaneously, the side effects can occur. Study of drug-protein interactions is thus important in the clinical therapy and in the development of new drugs.

In this study the displacement of L-tryptophan from its binding site on human serum albumin (HSA) by several drugs was investigated by capillary electrophoresis-frontal analysis (CE-FA). It was proved that L-tryptophan bonding was affected by another drugs when they were co-administrated. Therefore CE-FA method is suitable for competitive studies of concurrently administered drugs.

1 Introduction

HSA is the most abundant protein in blood plasma (60 % of total proteins of plasma) and it can be divided into three homologous helical domains (I-III) and each of these domains contain two subdomains A and B. HSA has two major drug binding sites Sudlow site 1 (IIA) and Sudlow site 2 (IIIA) [1]. It is illustrated in Fig. 1. One drug (displacer) may successfully displace another (target) from binding site on HSA when they are co-administrated in therapy. The increase of free drug concentration can cause the increase of side effects, even toxicity. Rate of displacement depends on the drugs concentrations and their relative affinities for the binding site. The drug displacement interactions can be studied by several methods, for example microdialysis, high performance liquid chromatography or circular dichroism [2]. In this study CE-FA method was used due to its many advantages which includes very low sample consumption, short analysis time and high efficiency. This method is robust, it can be used in high-throughput screening of drugs and moreover it is suitable for high and low affinity interactions [3].

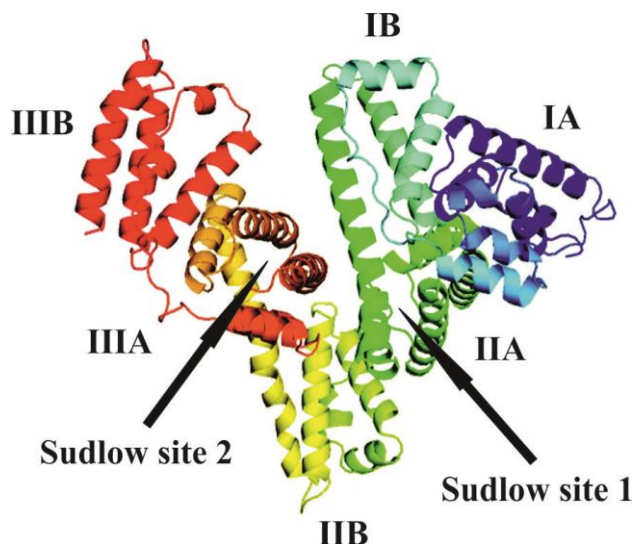


Fig. 1. The structure of human serum albumin.

2 Experimental

2.1 Instruments

The CE-FA experiments were performed on Agilent ^{3D}Capillary Electrophoresis System (Agilent Technologies, Waldbronn, Germany) with UV-VIS diode array detector. The fused silica capillary (Polymicro Technologies-Phoenix, USA) of 58.5 cm total length and 75 μm I.D. was used in experiments. Data were collected and analysed using ChemStation software (Agilent Technologies) and MS Excel 2010 (Microsoft, Redmont, WA, USA).

2.2 Chemicals

Investigated drugs (diclofenac, ibuprofen, chlorpropamide, tolbutamide), HSA, NaOH, boric acid and sodium tetraborate decahydrate were obtained from Sigma-Aldrich (Steinheim, Germany). L-tryptophan was obtained from Merck (Darmstadt, Germany), HCl was purchased from Fluka (Buchs, Switzerland).

2.3 Solution and sample preparation

The background electrolyte (BGE) was prepared by mixing 150 mM sodium tetraborate and 200 mM boric acid to resulting pH = 8.5, pH values were measured with an Orion Research EA940 (Orion Research-Waltham, USA).

Stock solutions of diclofenac and L-tryptophan (5 mM) were prepared in BGE, stock solutions of chlorpropamide and tolbutamide (5 mM) were prepared in BGE with 5 % (v/v) methanol, stock solution of ibuprofen (5 mM) was prepared in BGE with 100 μl 0.1 M NaOH. Working solutions were obtained from stock solutions by dilution with BGE to a desired concentration. Stock solution of protein (500 μM) was prepared daily in BGE.

2.4 Electrophoresis conditions

A new capillary was conditioned by rinsing with 1 M NaOH for 20 min, then with deionised water for 10 min and finally with the BGE for 20 min. At the beginning of each day, the capillary was conditioned by rinsing with 1 M HCl for 10 min, deionized water for 5 min, 1 M NaOH for 10 min, deionized water for 5 min and the BGE for 20 min. Before each analysis the capillary was flushed for 1 min with 1 M HCl, for 1 min with deionized water, for 2.5 min with 1 M NaOH, for 1 min with deionized water and for 2 min with BGE. The capillary was thermostated at 25 °C, samples were injected at 35 mbar for 40 s and the voltage of 14 kV was applied for separation. Detection wavelength was 220 nm.

3 Results and Discussion

Calibration curve was established from solutions contains variable amount of L-tryptophan in range 20-800 μM in absence of HSA. For competitive studies mixtures with increasing concentration (0-200 μM) of displacer and fixed L-tryptophan (100, 300 μM) and HSA (75 μM) concentration were used. Mixture containing L-tryptophan, tolbutamide and HSA was exception, due to the solubility of tolbutamide 25 μM concentration of HSA was used. Concentration of free L-tryptophan was measured from height of the plateau - H_f and calculated based on external standard in HSA absence. Electropherogram obtained by measuring sample containing L-tryptophan, diclofenac and HSA is illustrated in Fig. 2. Measurements showed that L-tryptophan was displaced from its binding site on HSA by diclofenac, ibuprofen, chlorpropamide and tolbutamide. These results correspond with information in literature [4-7].

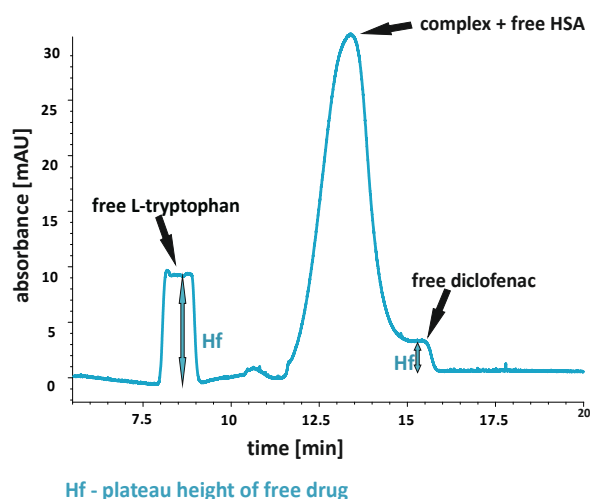


Fig. 2. Typical electropherogram of mixture containing L-tryptophan, diclofenac and HSA.

4 Conclusions

CE-FA method for study of competitive interactions of drugs with human serum albumin was developed. CE-FA has very low sample consumption, high resolution and it is suitable for high and low affinity interactions. Results proved that L-tryptophan

bonding was affected by another drugs. This information is in agreement with literature. Therefore CE-FA method is suitable for competitive studies of concurrently administered drugs.

Acknowledgement

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P47 PROTOTYPING OF A SIMPLE DEVICE FOR PARALLEL DOT BLOTTING USING CNC MACHINING

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Summary

Computer numerical control (CNC) machining has been tested for rapid prototyping of a new design of a dot-blotting device suitable for parallel testing of antibodies. The device, machined from polycarbonate, was prepared using an inexpensive computer controlled CNC router.

1 Introduction

CNC machining operated by commands coded in specific programming language is now available for inexpensive laboratory prototype machining. Computer-aided manufacturing (CAM) software is used to translate computer-generated designs (CAD, 3D model, bitmap, etc.) and convert the code into vectors. CAM software library must

contain precise definitions of all machining tools and bits including size, shape, purpose, safe load and so on. Vectors, together with tool parameters, define so-called toolpaths, which are then translated into code. Automation of the machining process allows for precise fabrication even by operators with very brief training.

2 Experimental

Blotting is a common method used in molecular biology, where a complex sample is separated by electrophoresis and transferred (blotted) onto a membrane using a vacuum or capillary forces. Sample spots, immobilized on the membrane, are visualized for specific detection, e.g. labeled DNA probe (DNA sample, Southern blot) or antibodies (protein sample, Western blot). Dot-blotting is a simplified method, involving direct application of several less complex samples directly onto the membrane.

The proposed new design of dot-blotter allows for sample-specific on-device washing and treatment in contrast with commercial devices, where the membrane has to be taken out of the device and submerged in washing and visualization solutions. The presented device was designed and programmed in Vectric VCarve CAD/CAM software and milled in polycarbonate and polyacetal using CNC router. Sample loading is provided by a vacuum chamber under the membrane while the on-membrane sample treatment is realized by drainage system also connected to a source of vacuum. Leaking is prevented by seals precisely tailored for the device using computer-controlled electronic cutter on a sheet of nitrile rubber.

3 Discussion

Fabrication methods can be divided into two groups: those where material is removed from the substrate are called subtractive, while those where product is built by adding material are called additive. As subtractive method, the CNC milling provides much shorter manufacturing times than additive techniques such as 3D printing when fabricating robust, sturdier objects. This speeds up the prototyping process as the unforeseen complications and design flaws can be mended in a matter of hours or minutes. On-device sample treatment provided by the new device enables parallel analysis of several dissimilar samples using different washing and visualizing agents for different samples. This represents step forward from the older dot-blotters where the whole sheet of membrane has to be treated at once. The new device is intended to be used mainly in verification of specificity, affinity and avidity of monoclonal and polyclonal antibodies.



Fig. 1. An example of the fabricated parallel dot-blotter.

4 Conclusion

CNC machining offers much freedom in designing and rapid prototyping of analytical devices. Instead of months or years of training, just a basic knowledge of the drafting software and basic computer skills are required to operate CNC machines. Accelerated evolution of the product provided by faster fabrication times allowed for more time spend experimenting with different functional and design concepts.

Acknowledgements

This work was supported by the grant 15-15479S from the Grant Agency of the Czech Republic.

P48 ANALYSIS OF SELECTED XENOESTROGENS IN NEOPLASTICALLY TISSUES USING COUPLED CHROMATOGRAPHIC TECHNIQUES

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1 Introduction

One of the most commonly diagnosed cancers in women is endometrial cancer. Searching for compounds that contribute to the development of cancer cells in the reproductive organs, we should pay attention to Zearalenone (ZEA) and its metabolites. They belong to the group of endocrine disruptors, as the structures of ZEA and its

metabolites are similar to estrogens (macrocyclic lactone ring) and show high affinity for estrogen receptors. They are particularly dangerous for women because they can interfere with the endocrine (hormonal) system. Zearalenone is absorbed within a short time after ingestion of contaminated food and rapidly metabolized in the gastrointestinal tract to more toxic compounds. The most dangerous metabolite is α -ZAL. Detection of these compounds in tissues requires using of highly sensitive methods. Xenoestrogens are present in tissues at low concentrations, so isolation and extraction methods must be very selective and specific [1-3]. In our research we made attempts to develop an efficient method for sample preparation and qualitative and quantitative determination.

2 Experimental

As the sample preparation method we decided to use the QuEChERS technique. As a starting point, we used the procedure proposed by Woźniak [4]. Following suggestions from literature [5,6] we modified the various stages of the QuEChERS, looking for high recovery and purification of the sample. In the first step, dSPE, we used different sorbents. We tested a variety of sorbents (PSA, Diamine, Aminopropyl, C18) in different quantities. High-performance liquid chromatography (HPLC) with fluorescence detection was used for identification and quantitative determination, and Ultra-high performance liquid chromatography (UHPLC) with tandem mass spectrometry for qualitative analysis. The optimal parameters of the detectors, stationary phase and mobile phase composition were selected for our analysis. The above procedure for the isolation and determination of the selected analytes was validated based on the following criteria: linearity, precision, accuracy, limit of detection and limit of quantification. The developed procedure was used to study neoplastic tissues of the uterus (endometrial cancer).

3 Result and Discussion

The HPLC-FLD method is linear in the concentration range 100-748 ng/ml with regression coefficients $>0,999$. LOQ for the analyzed compounds is at a satisfactory level (ZEA 82 ng/ml and α -ZEL 73 ng/ml). The UHPLC-QTOF/MS method is linear in the tested range of concentrations and has low LOQ (ZEA 0,10 ng/ml; metabolites < 30 ng/ml). Unfortunately, the matrix effect prevented running a quantitative analysis. Mass spectrometry was used to identify the compounds. The selected extraction method (QuEChERS technique) enabled us to achieve a satisfactory level of isolation, enrichment and purification of extracts for two compounds ZEA (R = 82%) and α -ZEL (R= 51%). The results of the analysis show that in 33 to 58 examined neoplastically changed tissues α -zearalenol was detected and quantified.

4 Conclusion

Isolating analytes from tissue is a challenge for the analyst because such matrix is highly heterogeneous. The developed version of the QuEChERS procedure successfully allows to isolate an examined compound and purify the sample by selected

sorbent. The presence of the tested compounds in the analyzed tissues confirms the tendency of xenoestrogens to accumulate in the reproductive organs.

Acknowledgement

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P49 HEXAHISTIDINE LABELING OF OLIGOSACCHARIDES

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Summary

We have studied various aminoacids and peptides as alternative labels for saccharide analysis. A hexahistidine peptide was a starting pattern for our experiments because this sequence is very well known as HisTag for the protein purification. The hexahistidine label allows fast analysis by capillary zone electrophoresis thanks to high number of positive charges of histidine molecules. Thanks to this approach the analysis requires two or three times shorter separation time in comparison of analysis of oligosaccharides tagged by one times charged labels.

1 Introduction

Today glycomics plays important role in the field of the bioanalytical chemistry. However, the analysis of glycans requires suitable methods because of the complex structure of glycans. Many techniques include the labeling of oligosaccharides for improvement of separation or detection properties. Attachment of a small molecule with high number of positive charges should provide fast migration in the capillary zone

electrophoresis and sensitive detection by the positive mode mass spectrometry. The hexahistidine well known from the HisTag technology was a natural choice. The histidine is easily protonated and is compatible with conditions of the positive mode electrospray ionization.

2 Materials and methods

Histidine (His), hexahistidine (6His), aminoacids (Arg, Lys) and peptides (5Gly3His, 2Gly6His) were used for labeling of oligosaccharides via reductive amination. The histidine with $pI = 7.59$ provided positive charge of labeled molecules in background electrolyte - acetic acid or formic acid and thus it allowed performing capillary electrophoresis of oligosaccharides in positive mode (positive voltage on the injection side of a capillary). Moreover, the histidine absorbs well the light at 214 nm and can be utilized for purification of labeled oligosaccharides on a boron column with UV detection. The oligosaccharides with different labels were tested with the capillary zone electrophoresis coupled with QTOF mass spectrometer.

3 Results and Discussion

Labeling of oligosaccharides by aminoacids and peptides in this study have in all cases led to sufficient separation by capillary electrophoresis. The migration times of labeled oligosaccharides were different depending on a number of charges provided by the label. The highest velocity provided oligosaccharides labeled by hexahistidine (2Gly6His). Maltooligooligosaccharides (isomaltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose) labeled by hexahistidine sequence migrated approximately two times faster than the same standards labeled by histidine alone (Fig. 1). It means that longer histidine-chain has positive impact on shorter analysis. We have tested also labeling of oligosaccharides by hexahistidine with and without a spacer for minimization of the steric exclusion and charge repulsion. The hexahistidine is usually prepared in sequence with two molecules of glycine but no impact on the labeling reaction was observed in this study. We have observed only one difference between 2Gly6His- and 6His-labeled oligosaccharides: the standards labeled by 2Gly6His migrated a little bit slower than the standards labeled by 6His since two molecules of glycine make molecule larger without bringing any additional positive charge. We have tested more complex samples such as glycans released by enzyme PNGase F from ribonuclease B. The separation of these glycans labeled by 2Gly6His was fast and sufficient and molecules created two, three and four times charged ions in mass spectrometric records.

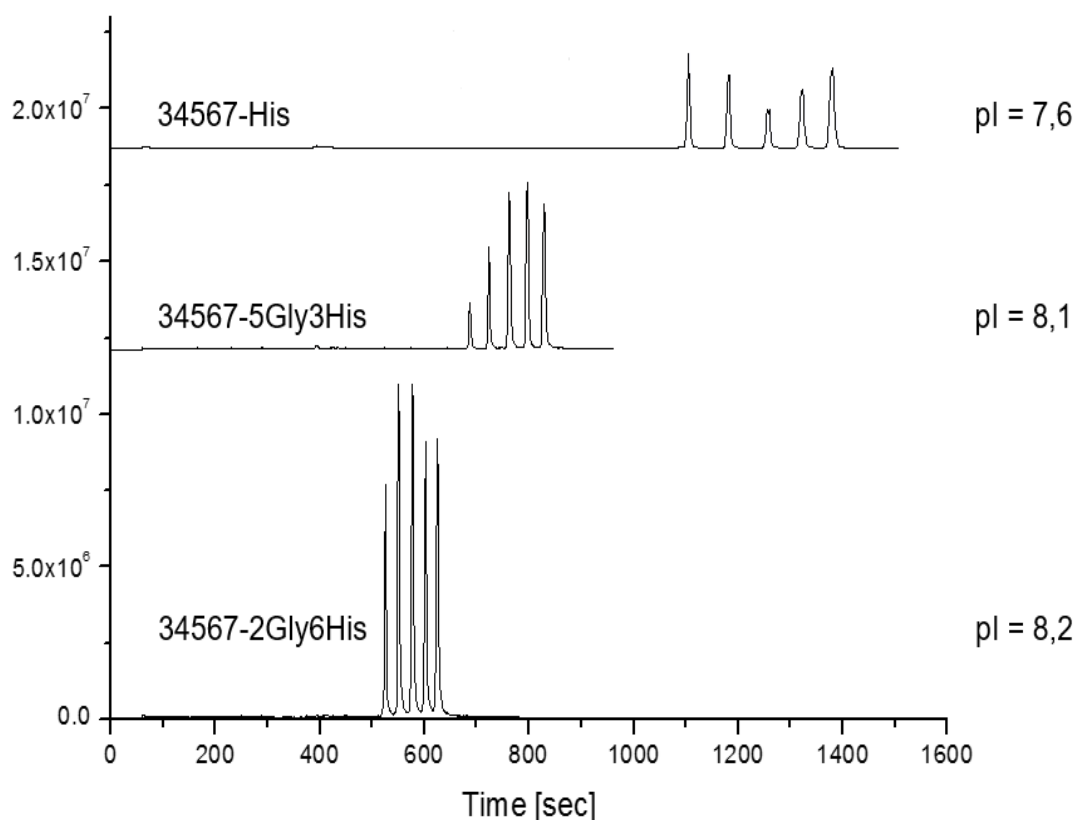


Fig. 1. CE/ESI/TOF-MS of maltooligosaccharides labeled by different number of histidines. BGE: 1M formic acid, 15 kV, bare fused-silica capillary, ID 50 μ m, 70 cm.

4 Conclusions

We have presented another approach for labeling of oligosaccharides suitable for capillary zone electrophoresis coupled with mass spectrometric detection. Oligosaccharides labeled by peptides with hexahistidine sequence have faster separation resulting in two or three times shorter time in comparison to oligosaccharides with singly charged labels.

Acknowledgement

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**P50 ON-A-CHIP ELECTROPHORESIS FOR THE
CHARACTERIZATION OF QUANTUM DOTS BIOCONJUGATES**

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Summary

On a chip electrophoresis was used to characterize quantum dots (QDs) and their conjugates with biological molecules. Due to very diverse and numerous applications of QDs, it is really important to make a tool for precise and controlled detection of QDs. In this study, we investigate on a chip detection and separation of QDs bioconjugated with BSA (bovine serum albumin) using homemade equipment based on principle of capillary electrophoresis (CE) and optical detection. Satisfactory separation of complex from free QDs was achieved with 10mM Borate Buffered Saline (BBS) aqueous solution (pH 9.14), as a separation buffer, within 10min.

1 Introduction

Semiconductor nanocrystal, also known as quantum dots (QDs), are nano-scaled inorganic particle in the size range of 1-10nm [1]. In last decade QDs find great application in medicine and biology usage, due to their photostability and resistance to chemical degradation [2]. By providing unique optical and magnetical properties QDs can facilitate detection and identification of different biomolecules such as protein, peptides and nucleic acids [3] QDs modified by BSA have been applied as ion sensors, fluorescence resonance energy transfer and chemiluminescence resonance energy transfer [4]. Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) has been used for characterization of QDs and their bioconjugates [5-7].

Aim of this work is to use fast and simple method for investigate bonding between QDs and biomolecules. Conjugation of QDs with bovine serum albumin (BSA) was achieved via covalent coupling using two step procedure combining 1-ethyl-3-(3-dimethylaminopropyl)carboiimide (EDC) with N-hydroxysulfosuccinimide (sulfo-NHS). In this work detection and separation of bioconjugated QDs was done in microfluidic system, using on chip electrophoresis and optical detection. Separation was done in PDMS chip, which was used due to its advantages: low cost, easy to fabricate, transparent and flexible, which makes it promising in device fabrication, allowing easier integration of electrodes and ports of microdialysis coupling [8]. By investigating interaction between QDs and biomolecules this research can lead to a better understanding of how QDs behave in cell environment, which is extremely important for their biological application.

2 Experimental

2.1 Preparation of QDs-BSA conjugates via EDC/NHS

Briefly, to the solution of CdTe QDs (200 μ l, 0.1 mg/mL) EDC (200 μ l, 50 mmol/L) and NHS (200 μ L, 5 mmol/L) were added and solution was then incubated at 32°C for 30 min. Then, BSA (200 μ L; 0.01, 0.05, 0.075, 0.1 mg/mL) was added to the solution and incubated at 32°C for 2 h while shaking [10].

2.2 Experimental set up

Capillary electrophoresis of QDs and QDs-protein conjugates was carried out using home built system “black box” (Figure 1a). The black box consists of the three parts: light source, sample holder and light detector. Excitation light is generated by ultraviolet light-emitting diode (UV LEDs) and filtered by optical 380 nm low pass filter. This light excites fluorescence of the sample and emitted light is selected by 420-680 nm high pass optical filter. This emitted light is detected by photodetector including photomultiplier.

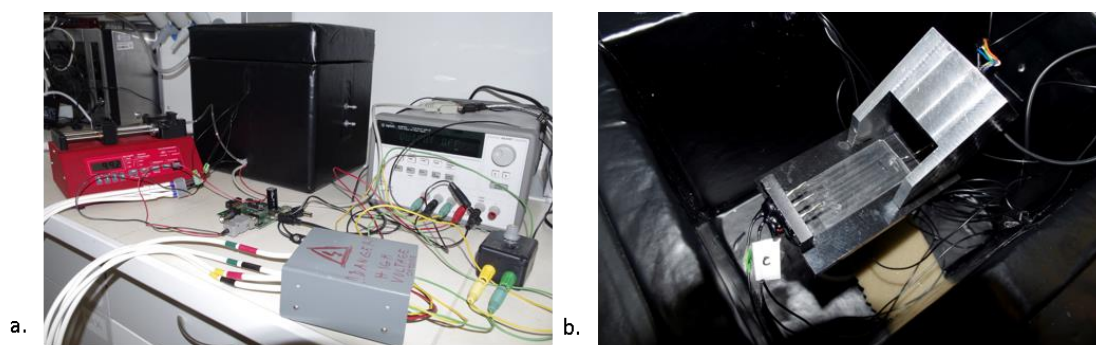


Fig. 1. a) Experimental set up: “black box”, syringe pump and power supplies, b) PDMS chip.

2.3 Fabrication of a chip and CE procedure

A high resolution printing is used to print design on a chrome transparency mask. This transparency serves as a photomask in photolithography to produce a positive relief of photoresist on silica wafer. Silica mold with channels of 50 μ m width was made and used for rapid molding of PDMS. The patterned side of the PDMS was treated with oxygen plasma (200 W, 15 min) and bonded permanently with a plasma-treated glass substrate to form a closed fluidic system (Figure 1b).

The applied voltage for the electrophoretic separation was 3 kV, and 10 mM Borate Buffered Saline (BBS) aqueous solution pH 9.14 was used as a background electrolyte. In the slight alkaline solution, CdTe possess negative charges due to dissociation of carboxylic groups on their surface and therefore migrate to the anode in the electric field.

3 Results and Discussion

Measuring the changes in the electrophoretic mobility overtime of bioconjugated QDs by CE we can determined binding of QDs with biomolecules, since interaction with these molecules will result in changes in the hydrodynamic size of QDs [3]. First, CdTe QDs capped with MPA (CdTe-MPA) were prepared in aqueous solution phase. The thiol group of MPA is linked to the surface of CdTe QDs by thiol group-Cd coordination, and the functional carboxylic group is free, which can be easily coupled to biomolecules with amino groups, such as proteins, peptides and amino acids. Coupling agent EDC/NHS was used to bioconjugate QDs with BSA. Figure 2 shows separation of free CdTe-MPA QDs from their protein conjugates. From results we can see that in alkaline buffer, the free QDs are well separated from QDs-BSA by CE on a chip within 10 minutes. The influence of protein concentration on fluorescence intensity was studied. It was noticed that fluorescence intensity of free QDs (QDs peak) is decreasing and fluorescence intensity of QDs bioconjugates (QDs-BSA peak) is increasing with increasing of protein concentration from 0 to 0.1 mg/mL. Decreasing and increasing of fluorescence intensities of free QDs and QDs-BSA complex with increasing of concentration of BSA shows a good linearity coefficient of determination $R^2=0.96$ and $R^2=0.98$ respectively.

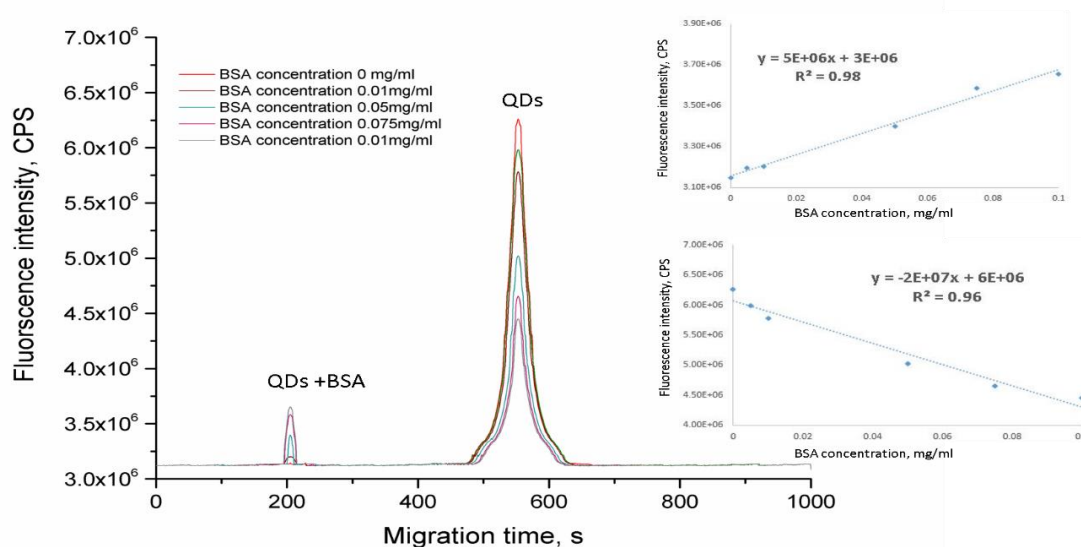


Fig. 2. Electropherogram of the CdTe-MPA QDs bioconjugated with various concentration of BSA, EDC/NHS as a coupling agent. Linear trend of QDs peak decreasing and QDs-BSA peak increasing with increasing of BSA concentration was also shown.

4 Conclusions

Quantum dots were conjugated with serum albumin protein and tested by electrophoretic separation. The free QDs are well separated from QDs-BSA by CE on

a chip within 10 minutes. Advantages of using this method are small reagents and sample requirements and short analysis time.

Acknowledgement

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P51 ON-CHIP COMPARTMENTALIZATION OF HIGH PRESSURE CHIP LC

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Summary

In this work microscale separation is combined with segmentation of the eluent flow. These two functionalities are monolithically integrated into a single glass chip. Analytes are encapsulated into numerous discrete volumes which are enclosed by the discontinuous oil phase. The compartmentalization of the eluent flow preserves the analytical resolution of the separation so further downstream processing such as derivatization or additional analysis is easily realizable.

1 Introduction

When combining droplet microfluidics with on-chip separation, the loss of individual band resolution can be prevented. This was first showed by Edgar *et al.* in their work describing the compartmentalization of electrophoretically separated components by means of droplet microfluidics [1]. Liquid chip chromatography was first combined with droplet microfluidics in a thermoset polyester (TPE) hybrid chip [2]. In the here presented work, we chose glass as material because chromatographic separations can

be performed under high pressure conditions (up to 300 bar). Due to the hydrophilic character of glass the channels have to be modified to obtain hydrophobic surfaces.

2 Experimental

The developed glass chips were fabricated in-house by common photolithography followed by a wet-etching procedure and high temperature bonding. To hydrophobize the channels the surfaces were activated by acid solutions and treated with a perfluorosilane.

High-pressure connection was provided by homemade steel connection clamps. In order to immobilize the column material two porous polymer frits were introduced into the channel via LED-assisted photopolymerization to retain the particles in between. Integration of the column was performed via a slurry packing method based on our previous work [3]. The mobile phase and oil flow were applied by syringe pumps.

3 Results and Discussion

Monolithic integration of high pressure chip-based LC with subsequent droplet generation was achieved using a T-junction design (Fig. 1+2). Four fluorescent analytes were separated under reversed-phased conditions and compartmentalized into a sequence of droplets using 70% acetonitrile/30% water (v/v) as mobile phase. All peaks are subdivided into numerous fractions by the oil phase perfluorodecalin. The droplets remain stable during transportation through the droplet channel (Fig. 3) and droplet frequencies of 10 Hz were obtained.

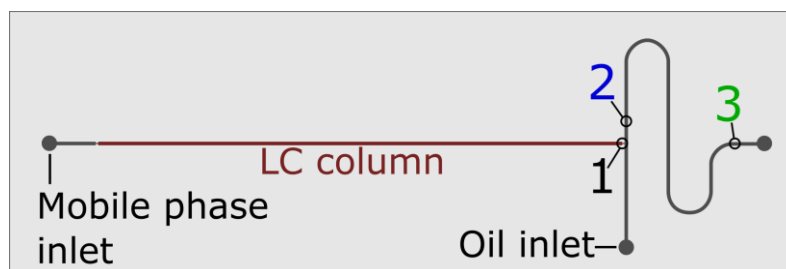


Fig. 1. Schematic drawing of the used chip layout. The column retaining porous frits were integrated at the beginning and the end of the column channel. Compartmentalization was achieved via a droplet generator directly positioned after the LC column (1).

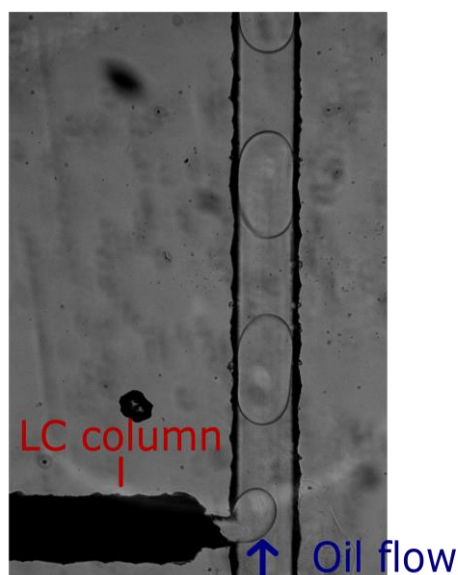


Fig. 2. Light-microscopic image showing the formation of segmented flow via a T-junction after the LC column.

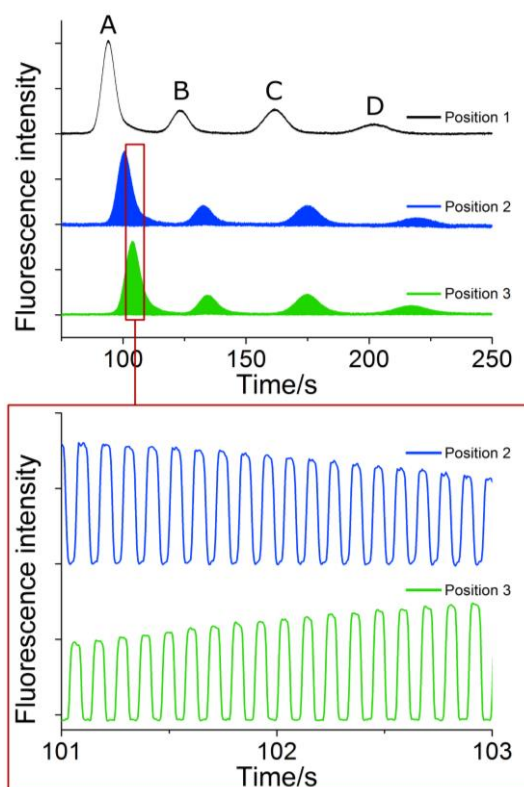


Fig. 3. Above: Chromatogram of a separation of (A) 7-Amino-4-methylcoumarin, (B) Anthra-cen-9-carbaldehyd, (C) Fluoranthene, (D) Benz-(a)anthracene at column effluent (Position 1), behind T-junction (2) and at the droplet channel outlet (3). ProntoSilC18 column 3 μm , column length: 5 cm mobile phase: 70 % acetonitrile 2,5 $\mu\text{L}/\text{min}$, back pressure 88 bar, oil flow: 2 $\mu\text{L}/\text{min}$. Below: Section of peak A illustrating the segmentation into droplets.

4 Conclusions

We demonstrated the use of droplet microfluidics for the compartmentalization of a chip-based LC separation. Detection of the separation at a later position resulted in an equal chromatogram proving that the chromatographic resolution is preserved within the droplets. Generation of the segmented flow had no disadvantageous effects on the separation performance.

Acknowledgement

We would like to thank Prof. Dr. Detlev Belder for his support and the opportunity to present the results of my master thesis on this conference. I want to give thanks to Renata Gerhardt M.Sc. for mentoring during my lab work of my master thesis.

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P52 RAPID DETERMINATION OF CRITICAL MICELLE CONCENTRATION BY TAYLOR DISPERSION ANALYSIS IN CAPILLARIES USING DIRECT AND INDIRECT DETECTION APPROACHES

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Summary

Critical micelle concentration belongs to the basic characteristic of surfactants. In this paper, the process of formation of micelles is studied by Taylor dispersion analysis (TDA) using capillary electrophoresis instrumentation. Two novel approaches based on indirect UV detection and indirect TDA with direct UV detection are introduced for the determination of CMC value of surfactant without any chromophore.

1 Introduction

Critical micelle concentration (CMC) which is the lowest concentration where micelles are presented in a surfactant solution belongs to the basic characteristic of micelle-forming compounds, i.e. surfactants. Generally, the determination of CMC is based on

changing physicochemical properties during the process of micelle formation including changes of conductivity, osmotic pressure, surface tension, or turbidity [1]. In this article, the next phenomena regarding the phase change of surfactants' monomers to micelles is reflected. Both objects (monomers and micelles) differ in their own diffusion coefficient what gives a possibility to determine the CMC value from measuring the change of the diffusion coefficient in their solution. In this context, the TDA is employed using a CE apparatus.

2 Experimental

All of the experiments were performed on the capillary electrophoresis instrumentation HP 3D-CE Agilent Technologies (Waldbronn, Germany) equipped with diode array detector (DAD). Uncoated fused silica capillaries (Polymicro Technologies, USA) of 50 μm i.d. with total length of 34 cm and effective length of 25 cm (to the DAD) was used. The Taylor dispersion analysis was performed by a pressure drop of 50 – 20 mbar. The TDA profiles were exported to *.csv files and analyzed. Sudan III in DMSO (0.1 mM) was used as a UV detection probe.

3 Results and Discussion

In the case of sodium octylbenzene sulfonate (SOBS), TDA using both frontal and pulse methodology was performed on samples with concentration ranging from 1.0 mM to 15 mM. Two linear regions with change in slopes were obtained. In the view of results, the CMC value of SOBS was estimated to be 9.7 ± 0.2 mM. The second studied surfactant, SDS, does not have any chromophore, hence, indirect UV detection should be applied. Theoretically, there are two possible ways: (i) to use an UV detection probe in a background electrolyte where the TDA will analyze negative peaks (or fronts) caused by the surfactant, or (ii) to use an UV detection probe only in a sample zone where the peaks (or fronts) for TDA are caused by the probe. In this study, SDS concentration range of 1.0 to 6.0 mM was tested by both frontal and pulse TDA using Sudan III as the UV detection probe. Both approaches, indirect and direct one, tested for the CMC measurements gave the change in slopes of diffusion coefficient dependence on SDS concentration. The CMC value of SDS surfactant was estimated to be 3.2 ± 0.3 mM.

4 Conclusions

In this study, the determination of CMC was addressed by the TDA with using CE instrumentation. This work shows that TDA has many possible uses and it is a unique tool in combination with the capillary electrophoresis instrumentation.

Acknowledgements

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P53 CLASSIFICATION OF REACTION BOUNDARIES AND THEIR USE FOR ON-LINE PRE-CONCENTRATION AND PRE-SEPARATION IN ELECTROPHORESIS

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Summary

The method for the on-line pre-concentration a pre-separation in electrophoresis using stationary reaction boundary was developed and verified.

1 Introduction

To reach higher sensitivity in electrophoresis we developed a convenient sample pre-concentration method which was on-line combined with common analytical ITP or CZE. Developed pre-concentration method is based on the principle of stationary reaction boundary. On such a stationary reaction boundary analytes are selectively focused and accumulated by electrokinetic dosing to the sharp, concentrated zone. After accumulation of sufficient amount of analyte, a focused zone is mobilized and transferred to the analytical column, where regular electrophoretic analysis is performed.

Tree basic types of the boundaries can be distinguished: neutralization reaction boundary – NRB (symmetrical and asymmetrical) and ligand step gradient boundary - LSGB.

The neutralization reaction boundary - simple NRB - can be created using only simple electrolytes; its pH span is symmetrical around pH 7.12. This type of boundary is convenient for the focusing of high and low MW ampholytes.

The asymmetrical NRB can be created using by addition of LMW ampholytes on one side of the boundary. This type of the boundary has lower span of pH and thus is more selective for the separation of ampholytes and/or is convenient for the extreme pH values pre-concentration e.g. of weak acids.

A stationary ligand step gradient - LSG - boundary is created in the column with two adjacent electrolytes branches of different chelating power and pH. It is convenient for the separation of metals.

The selectivity of the reaction boundaries can be influenced by a setting up the concentration, pH span and nature of the chelating agents. This way a substance of interest can be trapped on the boundary and most of ballast substances will pass through the system.

The examples of the analyses on the described boundaries are given; proteins, amino acids, weak acids, alkali earth and heavy metals were separated and pre-concentrated with one-to-two orders of magnitude accumulation.

2 Experimental

2.1 Equipment

The commercial apparatus (CS Isotachophoretic Analyzer, Villa Labeco, Slovakia) with two column arrangement and conductivity detection was used.

2.2 Procedure

A simple pre-concentration procedure was adopted, where a sample was continuously electrokinetically dosed from the large volume of dosing electrolyte in terminating chamber to the first pre-separation column, where was focused on the stationary reaction boundary. By changing composition of electrolytes focused zone is mobilized to the second analytical column and detected by e.g. ITP.

2.3 Electrolytes

2.3.1 Electrolyte system for the simple NRB

Alkaline (focusing) electrolyte: 0.01M NH₄Ac + 0.01M NH₄OH, pH=9.24

Acidic dosing (focusing) electrolyte: 0.01M HAc + 0.01M NH₄Ac, pH=4.75

ITP analytical electrolyte: LE: 0.02M NH₄Ac, TE: 0.01M HAc

2.3.2 Electrolyte system for ligand step gradient RB

Alkaline (focusing) electrolyte: 0.01M NH₄Ac + 0.01M NH₄OH + complexing agents (citrate, cresolphthalein complexone, methylthymol blue), pH=9.24

Acidic dosing (focusing) electrolyte: 0.01M HAc + 0.01M NH₄Ac, pH=4.75

ITP analytical electrolyte: LE: 0.02M NH₄Ac + 0.1M HAc + 0.002M ammonium citrate, pH=4.00, TE: 0.03M HAc

2.3.3 Electrolyte system for the asymmetrical NRB

Alkaline dosing electrolyte: 0.02M Histidine, pH = pI

Acidic (focusing) electrolyte: 0.02M HCl + 0.005M Histidine, pH = 1.7

ITP analytical electrolyte: 0.01 M HCl + Histidine pH=5.5, TE = 0,02M HAc

3 Results and Discussion

Example of pre-concentration of arginine and histidine from a model mixture using simple neutralization reaction boundary is on Fig.1.

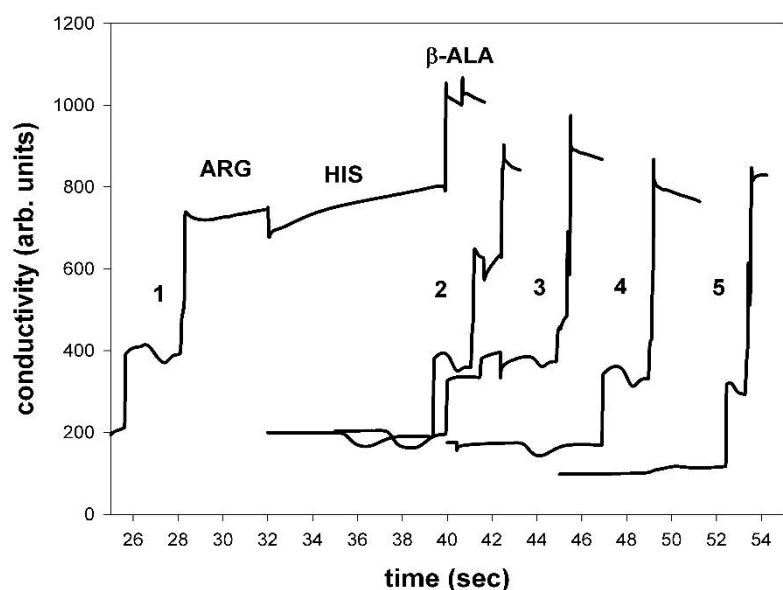


Fig. 1. ITP analysis of mixture using dosing time 1200 sec, at different concentration of AA (1- $2.5 \times 10^{-5} \text{Mol/l}$, 2- $2.5 \times 10^{-6} \text{Mol/l}$, 3- $2.5 \times 10^{-7} \text{Mol/l}$, 4- $2.5 \times 10^{-8} \text{Mol/l}$, 5- $2.5 \times 10^{-5} \text{Mol/l}$ – i.e. cLOD for ITP analysis without focusing.

Example of pre-concentration of metal chelates of Na^+ , Ca^{2+} , Mg^{2+} , Mn^{2+} , Cd^{2+} , Zn^{2+} , Ni^{2+} , Pb^{2+} , Cu^{2+} with subsequent on-line ITP analysis are depicted on Fig.2. The metal ions were continuously dosed into the column as a cations and they were selectively trapped (with except of sodium) on the stationary ligand field step gradient in the form of non moving zones of citrate complexes with effective zero charge.

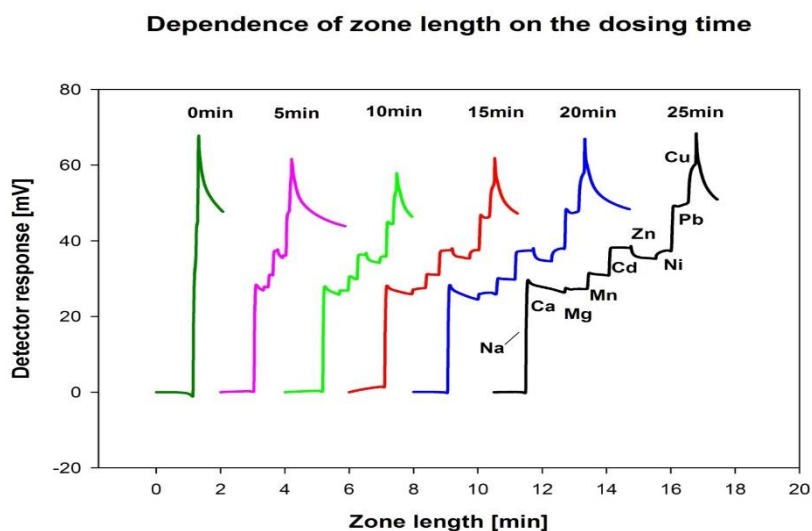


Fig.2. ITP analysis of metals at different dosing times, conc. $2.5 \times 10^{-5} \text{Mol/l}$ each.

Analysis of glyphosate using asymmetrical NRB

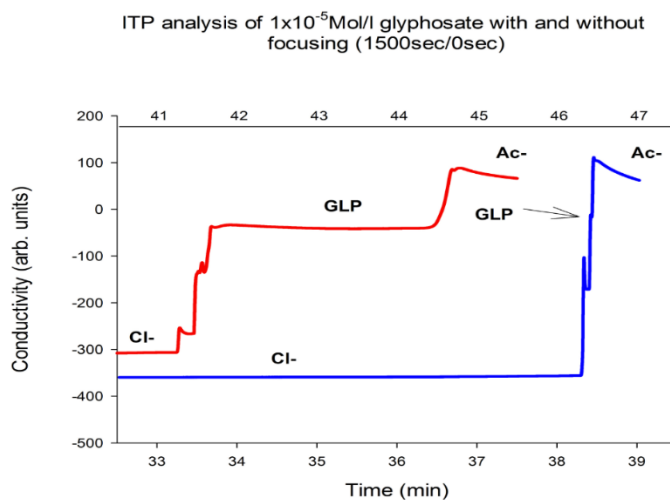


Fig. 3. Strong acid glyphosate was analysed by ITP without focusing (on the right) and with 1500 dosing (on the left).

4 Conclusions

The reached decrease in cLOD using focusing on different reaction boundaries is ca. two orders of magnitude lower in comparison to classical analysis, with acceptable increase of analysis time 20-25min. The boundaries can be tailored to focus different types of analyte, e.g. amino acids, metals and also acids.

P54 ELECTROCHEMICAL DEGRADATION OF IODINATED CONTRAST AGENTS USING BORON-DOPED DIAMOND ELECTRODE

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Summary

The aim of our work was to examine the potential of BDD electrodes in the electrochemical degradation of pharmaceuticals in waste waters. For this purpose, iodinated contrast agents were chosen for their frequent use in diagnostic procedures in modern medicine. The monitoring of degradation efficiency and identification of potential degradation products were performed using HPLC-HRMS technique.

1 Introduction

The presence of pharmaceuticals in the water environment and their potential negative effects on animals as well as human health had recently reached a lot of attention [1]. Thousands tons of these compounds are produced worldwide for usage in personal care products, human and veterinary medicine or agriculture. The occurrence of pharmaceuticals in the aquatic environment may arise from a direct usage, excretion, improper disposal of unused or expired pharmaceutical and feeding of animals [2, 3]. Many of these compounds are either toxic or resistant to a microbial degradation [4, 5]. Different physico-chemical methods are often used for the removal of pharmaceuticals from waste waters [3-5]. Among these methods, using electrochemical systems appears to be a promising solution of environmental problems [6]. Anodic oxidation is one of the most common electrochemical processes used for the waste water treatment [7]. The mechanism and efficiency of the electrochemical oxidation process of organic pollutants is influenced by the material of the anode. Boron-doped diamond (BDD) anodes are among the best materials for remediation of pollutants in water due to their properties such as stability and extremely high oxygen evolution, which leads to the formation of a large amount of hydroxyl radicals [4]. Identification of intermediates formed during the degradation process is of great importance because the degradation products may be more toxic than the parent compounds [8]. The most common used technique for the identification of degradation products is high-performance liquid chromatography in combination with mass spectrometry. High-resolution mass spectrometry (HRMS) analyzers enable to obtain information about molecule structure by combining sensitive full-spectrum data with high mass resolution and mass accuracy [9].

2 Experimental

The electrochemical degradation was performed using a heavily doped BDD electrode with an active area of 16 cm² (Institute of Electronics and Photonics, Faculty of Electrical Engineering and Information Technology, Slovak University of Technology in Bratislava) connected as an anode to a power supply GPS3005 (Gold Sun Technology, Taipei City, Taiwan). During the degradation process, solutions were stirred at 300 rpm using MR Hei-Standard stirrer (Heidolph Instruments, Schwabach, Germany). Temperature of solutions was controlled using infrared thermometer IR-77L (CEM, Shenzhen, China) and maintained below 30 °C. HPLC-HRMS measurements were performed using SPE-LC-Q-Exactive-MS (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

All chemicals used in this study were obtained from Merck (Darmstadt, Germany). The pharmaceutical product Omnipaque 350 (GE Healthcare, Nydalen, Norway) served as a stock solution of contrast agent, which contains 755 mg/mL of iohexol. The working solution with concentration of iohexol 1 mg/L was prepared by appropriate diluting.

Waste water composite samples were collected during 24 h from a waste water treatment plant (WWTP) Bratislava – Petržalka using an automated sampler device.

3 Results and Discussion

Iohexol is an iodinated, non-ionic, water soluble contrast agent used in diagnostic procedures in hospitals. Its chemical structure is showed in Figure 1.

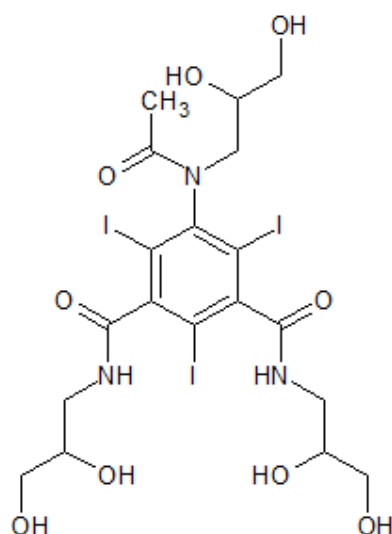


Fig. 1. Chemical structure of iohexol.

The first part of our work was to test the ability of the BDD electrode to degrade iohexol in the ultra pure water. For increasing of the conductivity, sodium chloride was added to the solution. Efficiency of the degradation process was monitored at two current densities, 30 and 100 mA/cm² in different times (0, 10, 30, 60 and 120 minutes). During the degradation process, the treated solution was stirred at 300 rpm and its temperature was monitored using an infrared thermometer. Similar experiments were performed in the waste water collected from WWTP Bratislava-Petržalka. In these experiments, the blank waste water was degraded as well as waste water with an addition of iohexol at the concentration 1 mg/L. Experimental conditions were the same as those which were used for the degradation of iohexol in the ultra-pure water.

The degradation efficiency was monitored using SPE-HPLC-MS technique. In the Table 1 concentrations of iohexol in ng/ml obtained after the given time of degradation at different current densities are shown. As we can see from data shown in the Table 1, if the current density 100 mA/cm² was used, the degradation efficiency of iohexol in the waste water was 97 %.

Table 1. Concentration of iohexol (ng/mL) obtained after the electrochemical degradation.

t [min]	spiked ultra pure water		spiked waste water	
	30 mA/cm ²	100 mA/cm ²	30 mA/cm ²	100 mA/cm ²
0	1100	1100	1400	1400
10	570	410	1000	1300
30	540	390	970	730
60	420	350	810	430
120	320	260	510	38

4 Conclusions

The degradation efficiency of proposed method was tested in spiked ultra pure water as well as in spiked waste water. The important part is the choice of optimal degradation conditions such as time and current density. From obtained results we can conclude, that the electrochemical degradation is a promising technique for removal of iodinated contrast agents from water environment.

Acknowledgement

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P55 ON-LINE CAPILLARY ELECTROPHORETIC METHOD FOR KINETIC AND INHIBITION STUDIES OF β -SECRETASE

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Summary

Alzheimer's disease (AD) represents the most common cause of dementia. According to amyloid cascade hypothesis, pathogenesis of the disease could be slowed down or even stopped by inhibition of β -secretase (BACE). On-line capillary electrophoretic (CE) methods represent a promising tool for screening of the enzyme's activity. A generic on-line CE method for kinetic and inhibition studies of BACE was therefore introduced.

1 Introduction

AD represents a severe neurodegenerative disorder characterized by progressive memory and cognitive functions impairment, with evolution to dementia and eventually death. Several prescription drugs treating symptoms of AD are currently available; however, none of them stops the progression of the disease itself. Since increased levels of amyloid β peptide of 42 amino acid residues ($A\beta_{1-42}$) was found in the brain tissue of the most of the patients, according to amyloid cascade hypothesis AD pathogenesis is stated by imbalance between $A\beta_{1-42}$ production and its clearance [1]. $A\beta_{1-42}$ subsequently aggregate into amyloid-derived diffusible ligands and amyloid plaques causing degradation of neurons. Specific inhibition of BACE, aspartic-acid protease responsible for $A\beta_{1-42}$ production, thus appears to be a promising way for slowing down or even stopping the progression of AD. CE represents a suitable analytical technique in this field due to high separation efficiency, minuscule sample and other reagents consumption, and high throughput via automation. Furthermore, a fused-silica capillary can be used as a nanoliter-scale reaction vessel. Such procedures are referred as on-line CE methods that comprise incubation of an enzymatic reaction and separation, detection and quantitation of reaction products in a single fully automated analysis. The main goal of this study was to introduce an on-line CE method for kinetic and inhibition studies of BACE.

2 Experimental

2.1 Capillary electrophoresis

An Agilent 7100 CE System equipped with a photodiode array UV-VIS detector at 200 nm was used for all experiments. The bare 75 μm id (375 μm od) fused-silica capillary was of 64.5 cm total length (56 cm effective length) and BGE was composed of 5 % acetic acid. Separations were accomplished by concomitant application of voltage (30 kV, positive polarity) and positive pressure towards anode (0.1 mbar) in the capillary

thermostated at 37°C. Data was acquired and peak areas were determined using the ChemStation software.

2.2 In-capillary reaction

All solutions of enzyme and reactants were prepared in incubation buffer consisting of 50 mM sodium acetate (pH 4.25). The injection procedure consisted of alternate introduction of four plugs of the model substrate and three plugs of BACE. Each plug was injected for 3 s into the capillary at a pressure of 30 mbar. The reaction mixture was incubated for 12 min at 37°C.

3 Results and Discussion

The method development, validation and the final approving were accomplished using model BACE's reaction with decapeptide SEVNLDAEFR as a probe substrate. An incubation buffer pH range of 3.75–5.5, BGE composition (acetic acid, ammonium acetate, sodium acetate) and its concentration range of 5–25 % and 30–70 mM, respectively, separation voltage range of 20–30 kV, and capillary temperature range of 23–50°C were tested in order to achieve maximal DAEFR moiety production yield. Since extensive numbers of candidate compounds are regularly screened within new drug development process, generic injection and mixing procedure based on transverse diffusion of laminar flow profile technique was adopted [2]. In principle, it consists of the alternate hydrodynamic introduction of four plugs of model substrate solution and three plugs of BACE. Due to the friction close to the inner capillary wall each plug possesses a parabolic profile and penetrates into the proceeding plug in the injection sequence, creating longitudinal interfaces between them. Reaction mixture is then quickly formed by transverse diffusion. Since diffusion represents an inherent property of all molecules any putative inhibitor of BACE's activity can be added into the reaction mixture and tested without need of additional assay changes. A typical electropherogram obtained with the optimized method is shown in Fig. 1.

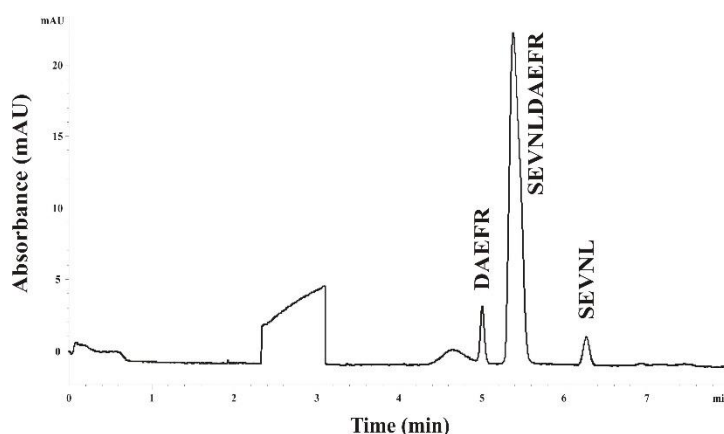


Fig. 1. Typical electropherogram obtained after the in-capillary reaction with 136 U/ml BACE and 250 μ M decapeptide SEVNLDAEFR; DAEFR and SEVNL are the pentapeptide products resulting from BACE reaction. Other incubation and separation conditions are summarized in Section 2.

When the optimization procedure was finished, the final method was validated and kinetic and inhibition studies were conducted in order to prove its practical applicability. The determination of the apparent Michaelis-Menten constant (K_m) and apparent maximum reaction velocity (V_{max}) was obtained with various amounts of the model substrate in the reaction mixture (50–1000 μM). Reaction velocity values were calculated in respect to incubation time of 12 minutes and the 5.7 $\mu\text{g/ml}$ concentration of BACE present in the reaction mixture. Data obtained were curve-fitted using non-linear regression analysis according to Michaelis-Menten model (see Fig. 2 Left). Inhibition study was carried out using the same incubation and separation conditions as the kinetic study; only model inhibitor was added to the substrate solution. For the half-inhibitory concentration (IC_{50}) assessment, various concentrations (0–10 μM) of KTEEISEVN-[Statine]-VAEF were used. The remaining BACE's activity values were plotted against the inhibitor concentration and evaluated using non-linear regression analysis according to model of competitive inhibition (see Fig. 2 Right). Apparent inhibition constant (K_i) value was then calculated using Cheng and Prusoff equation expressed as $K_i = IC_{50} / (1 + [S] / K_m)$ [3].

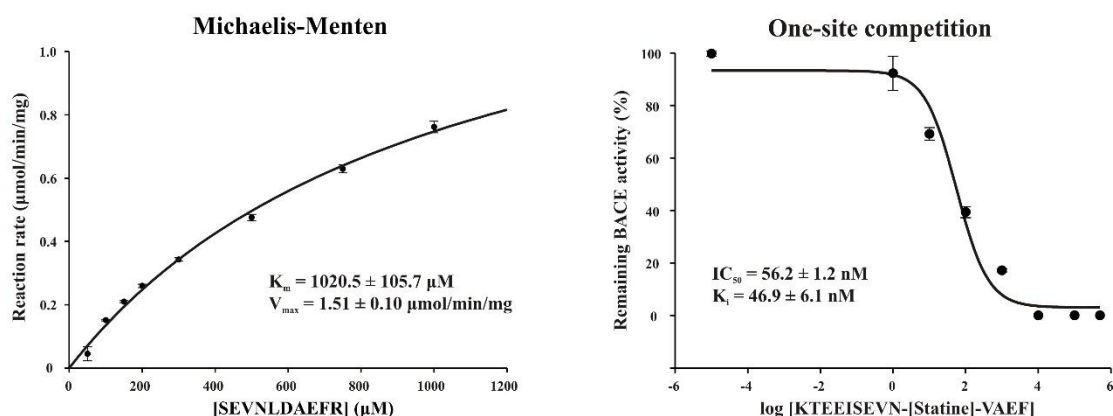


Fig. 2. Results of the conducted kinetic and inhibition study. Left: DAEFR moiety formation via BACE cleavage of the SEVNLDAEFR decapeptide. Right: Remaining BACE's activity plotted against inhibitor concentration with on-site competition fit. Incubation and separation conditions are summarized in Section 2 and 3, respectively.

4 Conclusions

An on-line method for studies of BACE's activity was developed. The obtained values of the basic kinetic and inhibition parameters are in good agreement with literature [4]. Furthermore, due to generic nature of diffusion-based mixing, presented method is directly applicable for screening of virtually any BACE inhibitor.

Acknowledgement

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P56 LIQUID CHROMATOGRAPHY WITH SURFACE-ASSISTED LASER DESORPTION/IONIZATION MASS SPECTROMETRY WITH SILVER NANOPARTICLES IN LIPID ANALYSIS

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Summary

This work aims at system optimization combining reversed phase liquid chromatography with on-line and off-line mass spectrometry detection. On-line detection was facilitated by APCI MS and off-line detection by SALDI MS using suspension of silver nanoparticles as matrix. Exploiting two different ionization mechanisms allowed obtaining complementary information from mass spectra of lipids. The application of the both techniques with high sensitivity and with low detection limits represents an alternative to GC-MS for analysis of nonpolar compounds.

1 Introduction

Lipids are hydrophobic or amphipathic molecules which can be divided into eight classes according to the Lipid MAPS consortium: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides. Typically, chromatographic techniques like gas or liquid chromatography coupled with mass spectrometry (MS) are used for lipidomic analysis of biological samples. Several chromatographic modes can be used in liquid phase separation, reversed-phase high performance liquid chromatography (RP-HPLC) is often used for nonpolar lipid separation according to the length of the fatty acyl chains and the number of position of double bonds. UV detection at low wavelengths is used for the detection of common glycerolipids (GL), but low sensitivity causes difficulties with their quantification. Advantageously, MS coupled to HPLC is a powerful tool for lipid analysis and provides good results in identification and quantification of glycerolipids.

Especially, atmospheric pressure chemical ionization (APCI) can be used for saturated as well as for unsaturated glycerolipids. A new approach relies on surface-assisted laser desorption/ionization (SALDI) with silver nanoparticles as matrix for lipid analysis.

2 Experimental

The separation was carried out on Zorbax SB-C18 column (100 x 2,1 mm; 3,5 μ m) using the HPLC system Agilent 1200 series (Agilent, USA). Mobile phase containing isopropanol and acetonitrile (50:50 v/v) was used in isocratic mode at a flow rate 0,4 mL/min. The UV detector wavelength was set to 206 nm. An APCI ion source in the positive mode was used for high-resolution MS analysis performed by system Agilent 6224 TOF LC/MS Series (Agilent, USA) and the operation parameters were set at as follows: nitrogen flow 10 L/min at 325 °C, vaporizer temperature 150 °C, nebulizer 40 psig, capillary voltage 4.5 kV, fragmentor voltage 70 V and skimmer voltage 65 V. Spectra were recorder in the m/z range 100-1700 [1].

Off-line SALDI detection was performed using the Autoflex speed TM MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) equipped with an Nd:YAG laser (355 nm). Lipids were analysed in the mass range m/z 300-1000 in the reflector positive mode. The recorded spectra were averaged from 4000 laser shots (80 x 50 shots per spot) and laser power was adjusted typically to 45 %. Suspension of silver nanoparticles (MesoSilver, Purest Colloids, Inc., USA) was used as matrix for SALDI MS experiments [2].

3 Results and Discussion

At first, optimization of HPLC condition for cholesteryl ester oleate (CHE) analysis was performed. The combination of online UV detection at 206 nm and APCI MS detection was employed after separation. A variety of mobile phases in different elution modes and condition of APCI ionization were tested to achieve short analysis time and spectra with all ions of interest (see Figure 1). A splitter combined with a laboratory-built spotter was used for eluent deposition on a target over a layer of silver nanoparticles for SALDI experiments. The suspension of silver nanoparticles was deposited on target inside a sub-atmospheric chamber to achieve a homogenous layer. Mass spectrometry imaging (MSI) was used for homogeneity control of silver nanoparticle distribution on the target. Low SALDI detection limit detection for CHE 7 ng/ml allowed determination of CHE in a sample of human plasma.

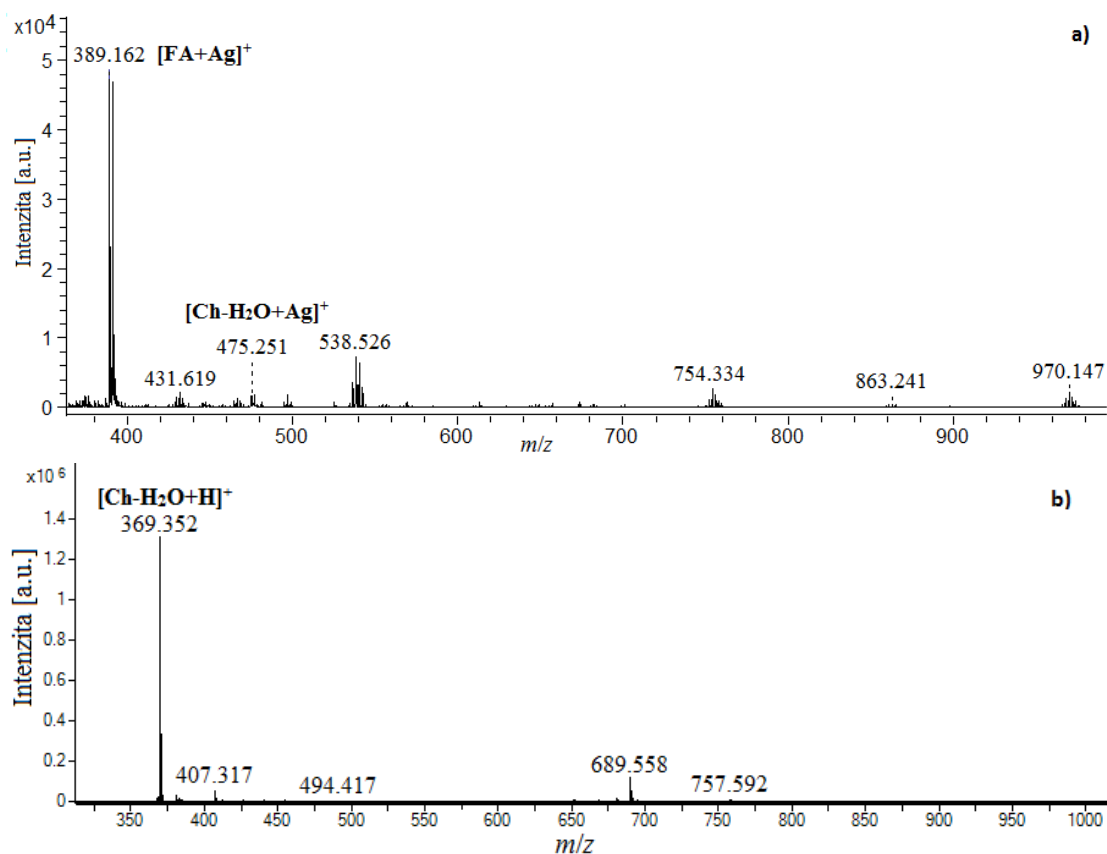


Fig. 1. Cholesterol ester oleate mass spectrum obtained by a) HPLC SALDI MS, b) HPLC APCI MS from single analysis.

Mass spectrometry with APCI ionization and SALDI with silver nanoparticles as supplementary techniques were applied for the determination of glycerolipids triolein (TO), tripalmitolein (TP) and tristearin (TS). Analyte characterization was based on comparison of identified fragment ions from spectra obtained by these two techniques. Characteristic fragment ions predicting the double bond position in the fatty acid chain were observed in SALDI spectra of TP and TO. The combination of the two methods with different ionization principles provided complementary information about the studied lipids.

4 Conclusions

A simple HPLC method with online eluent splitting was proposed for the analysis of cholesterol ester oleate in plasma samples by two different MS detection techniques. The optimization of conditions for RP-HPLC APCI MS and SALDI MS analysis of selected lipids, including the deposition of samples and silver nanoparticles, was accomplished. The combination of two complementary detection techniques may allow for better characterization of selected analytes in real samples.

Acknowledgement

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P57 FUNCTIONALIZATION OF SILVER NANOPARTICLES

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Summary

The aim of this work was to obtain biologically and chemically synthesized silver nanoparticles functionalized with antibiotic-tetracycline and to clarify the mechanism of the kinetic of tetracycline binding to silver nanoparticles. Functionalization of silver nanoparticles was confirmed by Fourier transform infrared spectroscopy (FTIR), total organic carbon (TOC), dynamic light scattering (DLS) and zeta potential measurements. We also evaluated the antimicrobial activity of biologically synthesized nanoparticles functionalized with tetracycline against range of Gram (+) and (-) bacteria strains.

1 Introduction

Antibacterial properties of silver have been known for centuries. With the development of nanotechnology it has been found that the silver nanoparticles possess significantly better antimicrobial properties. Growing problem of antibiotic resistance prompts the development of new antibacterial compounds and strategies. One of them is the use of AgNPs in association with antibiotic drugs. Many studies indicate that such combination enhances bactericidal effect. It was showed that antibacterial activities of biologically synthesized silver nanoparticles increase when are combined with antibiotics such as ampicillin or kanamycin [1, 2]. This observation suggests that silver nanoparticles functionalized with antibiotic can be potential drugs for the treatment skin and mucous membrane infections. Hence, the explanation of mechanisms of antibiotic

binding to silver nanoparticles and obtaining a stable complexes is necessary for future potent application as antibacterial agent.

2 Experimental

To study kinetic of binding tetracycline with silver nanoparticles two types of silver nanoparticles were used: (i) chemically synthesized stabilized with PVP and (ii) biologically synthesized by *Actinomycte* strain called CGG 11n [2]. Functionalized silver nanoparticles were prepared by mixing equal volumes of silver nanoparticles solution and tetracycline solution. The kinetic of the tetracycline binding process was investigated in batch adsorption experiments. Obtained silver nanoparticles were characterized by FTIR, TOC, DLS and zeta potential measurements. To compare the biocidal efficacy of functionalized and non-functionalized AgNPs, we determined zones of inhibition, minimal inhibitory and minimal bactericidal concentrations.

3 Results and Discussion

The kinetic of the antibiotic sorption by biologically synthesized silver nanoparticles showed that decrease of the antibiotics concentrations is not linear process and three separate steps can be identified: the initial rapid sorption step, gradual sorption step and last step of sorption equilibrium. Similar pattern was observed in the case of Ag-PVP, but in the last step instead of equilibrium occurs desorption. The maximum sorption effectiveness of nanoparticles CGG11n achieved in this experiment was 7.7% and for AG-PVP 12.56%. However, in the latter case the antibiotics in circa 50% were desorbed. Many physico-chemical analyzes confirmed tetracycline adsorption on the surface of silver nanoparticles. The size of both types of silver nanoparticles increases after functionalization which suggests that on the surface of silver nanoparticles is adsorbed layer of antibiotic. To determine the stability of functionalized silver nanoparticles, zeta potential analysis was carried out. For both types of silver nanoparticles the mean value of zeta potential slightly increased after functionalization but was still about -20 mV, which confirms their stability. The goal of the spectroscopic study was to detect changes in spectra of silver nanoparticles indicating the sorption of antibiotic. The FTIR spectrum of both types of silver nanoparticles showed obvious changes in the shape and the peak position before and after functionalization which suggests that surface of nanoparticles was modified with tetracycline. TOC analysis was another study that confirmed process of tetracycline adsorption on the surface of silver nanoparticles. After functionalization of Ag-CGG with tetracycline the amount of carbon adsorbed to the silver core increased. Both Ag-CGG as well as Ag-CGG functionalized with tetracycline exhibited antimicrobial activity against some pathogenic bacteria. Data from well-diffusion method and minimal inhibitory concentration showed that for *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Staphylococcus epidermidis*, Ag-CGG functionalized with tetracycline was more effective than alone Ag-CGG.

4 Conclusions

In our study we compared the kinetic of tetracycline sorption to biologically and chemically synthesized silver nanoparticles. Biologically synthesized AgNPs functionalized with tetracycline were more stable and showed better antimicrobial activity than non-modified analogue.

Acknowledgement

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P58 ARABIDOPSIS THALIANA MUTANTS DEFECTIVE IN SUBCELLULAR LOCALIZATION OF PIN AUXIN TRANSPORTERS

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Summary

Auxin is one of the essential regulators of plant development including organogenesis and its transport is mediated by PIN proteins. Polarity of PIN proteins is related to their activity that controls auxin distribution which is a key regulator for lateral root formation. We focused on candidates previously selected in forward genetic screen with obvious lateral root formation (LRF) defects that were also exhibiting agravitropic growth. We characterized them on the cellular level by monitoring the expression and polarity of PIN2 protein using immunocytochemical technique for whole-mount in situ protein localization in plants. We observed differences in PIN2 patterns of mutants and wild type.

1 Introduction

Auxin is one of the most important hormones in higher plants usually referred as growth hormone. It has essential role of regulating developmental processes like embryogenesis, organogenesis, tissue differentiation and tropic responses [1]. Plant tropisms as growing toward or away from stimulus such as light or gravity is reaction of one side organ elongation than the other resulting in a curvature toward or away from stimulus [2]. These responses require the coordinated and asymmetric distribution of auxin that depends on PIN-FORMED proteins (PIN proteins) localization [3]. PIN proteins are auxin-efflux transporters and play an important role in defining auxin gradients [4]. According to phylogenetic analysis PIN family can be divided into two subclasses, the plasma membrane localized PIN1-like (PIN1, 2, 3, 4, 7) and the endoplasmic reticulum localized PIN5-like (PIN5, 6, 8) proteins [5]. In our study we focused on PIN2 proteins as known auxin transporters mediating the auxin maximum and auxin redistribution responsible for gravitropic root growth [1]. For further characterization of previously selected agravitropic mutants we performed immunolocalization of PIN2 proteins to detect their expression and pattern in epidermal and cortical root cells.

2 Experiment and methods

2.1 Genetic screen based on NAA treatment

The application of exogenous auxin levels promotes pericycle cell division and induces *de novo* organ lateral root formation in the pericycle cells in wild type plants [6]. We designed a screen based on NAA – 1-Naphtaleneacetic acid (synthetic plant hormone from auxin family) treatment. The aim was to select candidates showing defects in LRF after NAA treatment compared to plants developing in control growth conditions.

2.2 Immunolocalization of PIN proteins

Some of the previously chosen candidates exhibited agravitropic root growth. To monitor the expression and polarity of PIN2, the auxin efflux facilitator associated with agravitropic growth, we performed whole mount immunolocalization following the protocol by Sauer et al. [7]. To detect PIN2 protein we used α -PIN2 rabbit primary antibody in dilution 1:1000 and Cy3 anti-rabbit secondary antibody in dilution 1:600. The procedure was automated by INTAVIS InsituProVSi Immuno Robot (<http://www.intavis.com/products/automated-ish-and-ihc/insituprovisi>) and subcellular protein visualization was monitored by Zeiss 700 confocal microscope (http://www.mikroskop.com.pl/pdf/LSM700_1.pdf).

3 Results and Discussion

Our aim was to select auxin-related mutants and identify the mutations in genes related to the auxin pathway resulting in changed protein function. Considering the agravitropic phenotype of selected candidates we decided to characterize them on the subcellular level by checking the expression and localization of PIN2 proteins. In wild type plants PIN2 proteins are localized in the apical side (shoot-apex facing) of the epidermis cells and basal side (root-apex facing) of the cortical cells [8]. Apical-polar

localization of PIN2 proteins in root epidermal cells is required for asymmetric auxin translocation, which effects gravitropic root growth [9]. Compared to wild type plants we observed different patterns of PIN2 proteins in the mutants in both epidermal and cortical root cells. Regarding the observations, we can predict that the chosen candidates possess alterations in the genes coding for regulators related to the PIN localization and auxin pathway.

4 Conclusions

We chose immunolocalization method to visualize PIN2 proteins in previously selected candidates with clear phenotype after NAA treatment and agravitropic root growth. The observed differences compared to wild type in the apical-basal polarity of PIN2 proteins helped us to select the best candidates for our later studies. We are continuing to characterize these mutants by performing phenotypic analysis and using molecular biology techniques including full genome sequencing to identify the corresponding genes.

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P59 COMBINATION OF TWO CAPILLARIES WITH DIFFERENT INTERNAL DIAMETERS FOR FAST SEPARATIONS

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Summary

High-speed capillary zone electrophoresis separation in a system formed by combining 50 and 100 μm capillaries is described. High electric field intensities were tested for chiral separation of R,S-1,1'-binaphthalene-2,2'-diyl hydrogen phosphate (BNP). Isopropyl derivative of cyclofructan (IP-CF6) in 500 mM sodium borate pH 9.5 was found to be optimal allowing 12-fold shorter separation time.

1 Introduction

Under normal conditions, separation of enantiomers in CE lasts for 5 to 30 minutes. However, this is too long for many applications. Substantially shorter separation times can be achieved e.g. by adjusting the length of separation path, injection from the short end of the capillary or pressure-assisted CE. But these techniques decrease the value of effective voltage along the separation capillary. Hence, it is better to use a system formed of two connected capillaries with different internal diameters for fast separation on a standard CE system [1]. This connection does not cause the decrease of effective voltage and it affects the separation time, too. In our work, we addressed the problem of accelerating the separation of enantiomers in capillary electrophoresis. In the future, we are interested in application of nanoparticles, e.g. quantum dots or magnetic nanoparticles, with chiral selectors in these systems.

2 Experimental

All experiments were performed on the capillary electrophoresis HP 3DCE, Agilent Technologies (Waldbronn, Germany) with a DAD detector. Bare fused-silica capillaries (Polymicro Technologies, USA) with a total length of 32.6 cm and effective length of 8.4 cm, and inner diameters of 5 to 250 μm were used. The technique of joining two capillaries with different internal diameters developed by Pavlíček et al. [2] was used to prepare connected capillaries. Here, the separation part of the capillary with length of 9.7 cm, and the auxiliary part of the capillary with length of 22.9 cm was connected.

3 Results and Discussion

BNP atropoisomers are negatively charged due to their dissociation in the basic electrolytes and they migrate as anions in an electric field. First, we studied combination of capillaries with different IDs. Here, we found the most reproducible way for

analysis/separation of R,S-BNP is connection of two capillaries with IDs of 50 μm and 100 μm . The next step covered optimization of separation conditions including buffer pH and concentration and IP-CF6 concentration of in connected capillaries. The most promising BGE was 500 mM sodium borate pH 9.5 with 60 mM IP-CF6. It provided the shortest time for separation of R,S-BNP (85 s at -5 kV).

4 Conclusions

The technique of joining two capillaries with different internal diameters is not time consuming. Connected capillaries prepared by this method can be used for successful and fast chiral separation. In comparison with 50 μm capillary and injection from the standard (“long”) end of the capillary, the coupled capillaries allow about 12-fold shorter separation time with resolution values greater than 1.5. The pilot results with addition of nanoparticles seem to be very promising.

Acknowledgements

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P60 2D DISTRIBUTION MAPPING OF CdTe QUANTUM DOTS INJECTED ONTO FILTRATION PAPER BY LASER-INDUCED BREAKDOWN SPECTROSCOPY

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Summary

In this study, the feasibility of Quantum dots (QDs) 2D distribution mapping on the substrate by Laser-Induced Breakdown Spectroscopy (LIBS) was examined. The major objective of this study was to describe phenomena occurring after applying aqueous solutions of QDs onto filtration paper. Especially, the influence of pH of QDs solutions on LIBS signal was investigated.

1 Introduction

LIBS has been widely used in modern analytical chemistry. It is very popular because of its advantages such as fast turn-around time, multi-elemental analysis (detection of light elements and halogens), analysis of samples in any state of matter, practically no sample preparation and possibility of remote sensing. On the top of this all, the main advantage is the possibility of elemental spatial distribution investigation [1-3]. Very novel is the idea of easy detection of different NPs types as was already successfully demonstrated in few papers [4-10].

In our study, QDs were prepared by formation of nanosized semiconductor particles in a so called “one-pot” colloidal synthesis. CdTe QDs capped by MPA (3-mercaptopropionic acid; MPA-QDs) were made. Basic properties of QDs were examined before main experiments. The size of these QDs was determined by Dynamic Light Scattering (DLS) and accurate concentration of Cd in QDs was determined by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES).

In this work, new interesting relations between changes in pH of aqueous QDs solutions and LIBS signal were investigated in detail. The results imply the possible use of QDs' sensitivity to external influences in paper chromatography. Also we prove that LIBS is a promising alternative to fluorescence methods as it enables detection of both non-luminescent QDs and QDs whose luminescence is affected by external conditions, such as pH change.

2 Experimental

2.1 Quantum dots

One type of CdTe QDs were prepared – MPA-QDs. Prepared MPA-QDs NP's contained (23.19 ± 0.06) % m/m of cadmium. The nominal average particle size was 8-10 nm (DLS), zeta-potential was (-45.0 ± 10.0) mV. Cadmium content of QDs was measured by ICP-OES spectrometer iCAP 6500 Duo (Thermo, UK).

2.2 LIBS

2.2.1 Apparatuses

A modified laser system UP-266 MACRO (New Wave, USA) equipped with software-controlled movement in x and y directions was used. The prepared filtration papers were placed onto a lab-made holder. Plasma emission was collected and transported by means of a 3m long optical fiber into the entrance of a monochromator TRIAX 320 (JobinYvon, France) and detected with the ICCD detector PI MAX3 (Princeton, USA). Pulse energy was set to 10 mJ, the delay of the signal detection after laser pulse was 0.8 μ s and the integration time was 5 μ s. The raster covered 15*15 points with 500 μ m spacing while laser spot diameter was approximately 100 μ m. Each emission spectrum was acquired in a single-shot mode on fresh spot. Parameters for measurements were selected based on our previous experiments and only minor modification was done [6].

2.2.2 Data evaluation

The intensity of analytical emission line Cd I at 508.58 nm was evaluated as maximum line intensity after appropriate background subtraction and the construction of 2D maps

showing the spatial distribution of Cd (i.e. QDs) in the spot of injected NPs were constructed. Each map consisted of 225 single pulse measurements obtained from the raster. All evaluations were conducted in R program [11].

3 Results and Discussion

The pH of prepared QDs aqueous solutions ($c=4$ mg/mL) was adjusted by 1M-HCl solution. The initial pH MPA-QDs solutions was 9.8. The pH of QDs solutions was changed and 1 μ L samples were dropped onto the paper in four replicates for pH range from 9.8 to 1.3. All spots were analyzed by LIBS mapping.

LIBS-maps of MPA-QDs were compiled in respect to variations pH of the solution ranging from 9.8 to 1.3 (Fig. 1), these maps show that QDs aqueous solutions are very sensitive to any change of pH and that LIBS method is able to note the change in the distribution of NPs in spots.

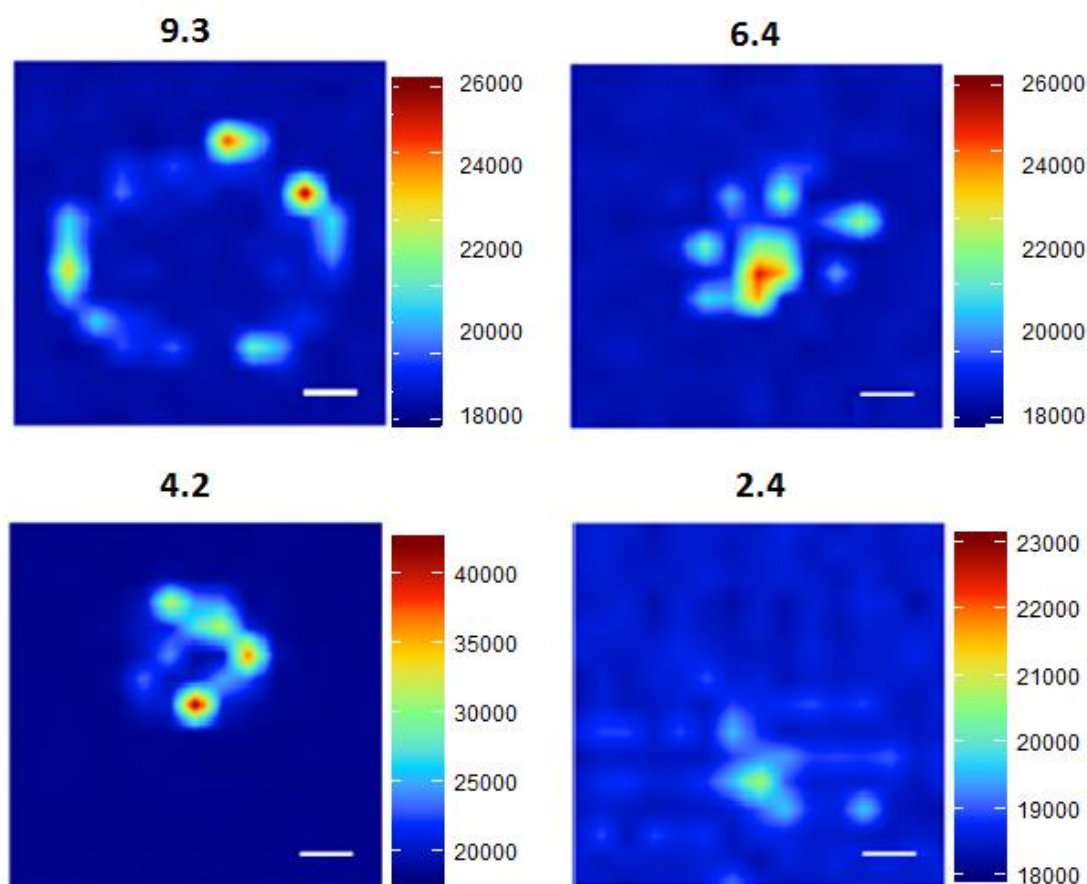


Fig. 1. MPA-QDs: LIBS maps for pH range QDs solution. Measurements were carried out for emission line Cd I at 508.58 nm. The scale shows 1000 μ m. Four pH values (9.3, 6.4, 4.2 and 2.4).

4 Conclusions

A major goal and novelty of this study is in the description of pH effect that have an impact on intensity of LIBS signal as well as spatial distribution of QDs on substrate – filtration paper, i.e. the adsorption phenomena occur after application of QD's aqueous solutions onto paper. In our opinion, depicted behavior of QDs has a significant potential to be used in paper chromatography as for their immobilization. The spatial distribution of QDs injected onto filtration paper is affected by pH of QDs solution, as it is clearly visible in the compiled LIBS maps.

Throughout this study was demonstrated that LIBS technique is a promising alternative to fluorescence methods since it enables detection of both non-luminescent QDs and QDs whose luminescence is affected by experimental conditions, e.g. pH, presence of other metal ions.

Acknowledgement

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**P61 CAPILLARY ELECTROPHORESIS WITH CONTACTLESS
CONDUCTIVITY DETECTION FOR RAPID DETERMINATION OF
MELDONIUM IN URINE**

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Summary

A simple, rapid and cheap capillary electrophoresis-capactively coupled contactless conductivity detection (CE-C⁴D) method for determination of meldonium (MEL) in urine samples was developed. Sample pretreatment was minimized to dilution of urine samples with DI water and MEL was determined by CE-C⁴D in background electrolyte consisting of 2 M acetic acid (pH 2.3) in ng mL⁻¹ to mg mL⁻¹ concentration range. Sample-to-sample analysis time was less than 4 min, limit of detection and quantification was 0.015 and 0.05 µg mL⁻¹, respectively, and the method showed excellent linearity ($r^2 \geq 0.9998$), recovery (97.6–99.9%) and intraday as well as interday repeatability (RSD ≤ 4.7%).

1 Introduction

The chemical name of meldonium (MEL) is 3-(2,2,2-trimethylhydrazinium) propionate dihydrate and MEL is an analogue of carnitine. Since January 1, 2016, MEL has been added to the World Anti-Doping Agency (WADA) list of banned substances because of “evidence of its use by athletes with the intention of enhancing performance” [1,2], and is classified as a metabolic modulator. Analytical determination of MEL is not suitable by analytical techniques with conventional UV-Vis absorbance detection due to the lack of UV-absorbing chromophores in its structure. In this contribution simple, rapid and cheap analytical method for determination of meldonium in urine samples was developed. The method requires minute volumes of urine sample, minimum sample treatment, is suitable for determination of ng mL⁻¹ to mg mL⁻¹ concentrations of MEL, is robust and accurate and is suitable for long-term analyses of urine samples without deteriorated analytical performance.

2 Experimental

7100 CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with a C⁴D (Admet, Prague, Czech Republic, operated at 50 V_{pp} and 1.84 MHz) and background electrolyte consisting of 2 M acetic acid at pH 2.3 were used for CE separation of MEL from compounds present in urine samples.

3 Results and Discussion

BGE solutions consisting of acetic acid were chosen for CE-C⁴D separation of MEL from urine cations, which might potentially interfere with determination of MEL.

Before injection, each urine was diluted 1:9 with DI water and two aliquots were prepared. One was used without any further adjustments, the other one was spiked with $1 \mu\text{g mL}^{-1}$ of MEL and the samples were analyzed in BGE solutions consisting of different concentrations of acetic acid. No co-migration of MEL with any matrix component was observed in BGE solution consisting of 2 M acetic acid for 10 unique urine samples. The total analysis time at optimized conditions was less than 4 min, including capillary flushing (40 s), sample injection (10 s) and MEL separation (180 s). A CE-C⁴D electropherogram of urine sample spiked with $0.25 \mu\text{g mL}^{-1}$ MEL including identification of all major inorganic cations (NH_4^+ , K^+ , Na^+ , Ca^{2+} and Mg^{2+}), Crea and His is shown in Figure 1.

CE-C⁴D analyses of MEL in non-spiked urine and in the same urine spiked at higher concentration levels (2 and $20 \mu\text{g mL}^{-1}$) are depicted in Figure 2. This Figure shows that reduction of injection volume by a factor of 5 (shortening of injection time from 10 to 2 s) fully eliminates possible co-migration of the higher MEL concentrations with an unknown matrix cationic compound at migration time of 2.81 min.

Excellent linearity ($r^2 \geq 0.9998$) was obtained for two concentration ranges, $0.02\text{--}4 \mu\text{g mL}^{-1}$ and $2\text{--}200 \mu\text{g mL}^{-1}$, by simply changing injection time from 10 to 2 s without the need for additional dilution of urine samples. Limit of detection was $0.015 \mu\text{g mL}^{-1}$ and average recoveries from urine samples spiked at $0.02\text{--}123.5 \mu\text{g mL}^{-1}$ MEL ranged from 97.6–99.9%. Repeatability of migration times and peak areas was better than 0.35% and 4.2% for intraday and 0.95% and 4.7% for interday measurements, respectively.

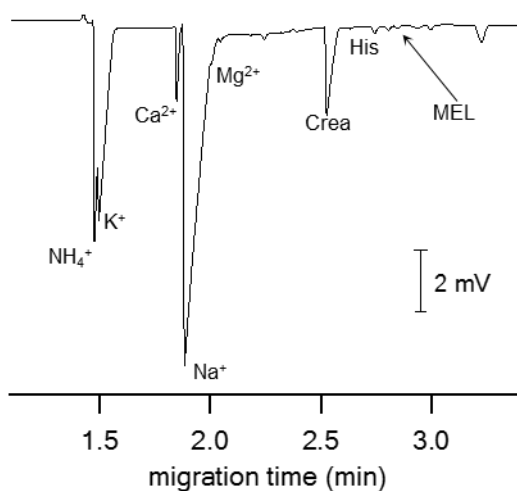


Fig. 1. CE-C⁴D analysis of 1:9 diluted urine spiked with $0.25 \mu\text{g mL}^{-1}$ of MEL. CE-C⁴D conditions: BGE, 2 M acetic acid (pH 2.3); injection, 50 mbar for 10 s; voltage, + 30 kV; capillary, $25 \mu\text{m}$ ID and $L_{\text{tot}}/L_{\text{eff}} = 55/42$ cm.

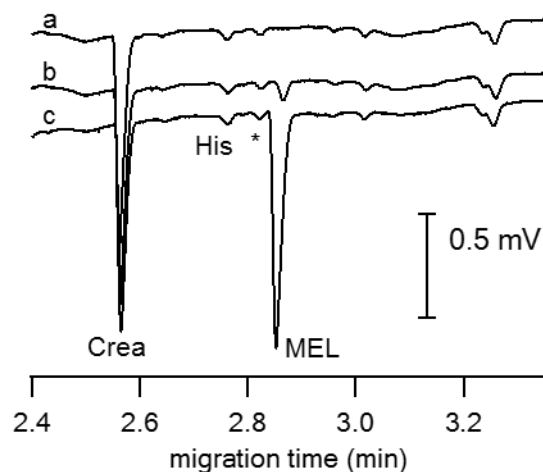


Fig. 2. CE-C⁴D analyses of 1:9 diluted urine sample without and with spiking by MEL. Diluted urine (a) and diluted urine spiked with 2 (b) and 20 (c) µg mL⁻¹ of MEL. CE-C⁴D conditions as for Figure 1 except for injection, 50 mbar for 2 s. * – unknown matrix cationic compound.

4 Conclusions

Simple, rapid and cheap CE-C⁴D method for determination of MEL in urine samples was developed. Minimum sample treatment, negligible consumption of samples, high sampling frequency and low cost are the major advantages of the developed method. The above reported advantages might be especially beneficial in analyses of large batches of urine samples associated with the actually increased need for determination of MEL and for initial testing of MEL in urine samples.

Acknowledgement

Financial support from the Czech Academy of Sciences (Institute Research Funding RVO: 68081715) and the Grant Agency of the Czech Republic (Grant No. 16-09135S) is gratefully acknowledged.

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P62 **BICARBONATE DUAL SENSOR BASED ON EU(III)
COMPLEX**

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Summary

This work describes the study of the formation of ternary [Eu(III)DO2A] and [Eu(III)DO3A] complexes with “antenna” picolinate-like ligands. The luminescent and electrochemical properties of these ternary Eu(III) complexes can be used for the detection of various organic ions and these properties can be utilized in order to develop a new dual sensor for ion analysis. In this work, the Eu(III) complexes were used as probes for the determination of bicarbonate ions in solution.

1 Introduction

Macrocyclic hexadentate (1,4,7,10-tetraazacyclododeca-1,7-diacetic acid, H₃DO2A) and heptadentate (1,4,7,10-tetraazacyclododeca-1,4,7-triacetic acid, H₃DO3A) ligands form Eu(III) complexes of high thermodynamic stability and kinetic inertness [1]. The addition of an “antenna” picolinate-like ligand to these complexes enhances their luminescent properties. These complexes are then able to react with other mono- and bidentate ligands (*e.g.* F⁻, acetate, carbonate, *etc.*) by substitution of the picolinate-like antenna to form more stable ternary adducts and it causes a decrease of luminescence analytical signal that is proportional to the concentration of the ions in solution. The differences in the stability constants of these ternary complexes can be utilized for electrochemical/luminiscent selective determination of anions [2, 3].

2 Experimental

Sodium bicarbonate, picolinic acid (PA), dipicolinic acid (DPA) (all p.a. grade, Sigma-Aldrich, USA) and isoquinoline-3-carboxylic acid (IQCA, Acros Organics, Belgium) were used as received. The macrocyclic ligands, H₃DO3A and H₂DO2A, were purchased from CheMatech (France). Stock solutions of EuCl₃ were prepared by dissolving Eu₂O₃ (p.a. Alfa, Germany) in a small excess of hydrochloric acid and were standardized by chelatometric titration. Luminescence spectra were recorded on the Quantamaster 300 Plus spectrometer (PTI Canada) with a flash Xe-lamp in the wavelength range 200-800 nm.

3 Results and Discussion

Eu(III) ternary highly-fluorescent complexes consisting of H₃DO3A and picolinate-like “antenna” ligands (L) ([Eu(DO3A)(L)]⁻) (see upper Fig. 1) react with carbonate anion

in order to form more stable non-fluorescent $[\text{Eu}(\text{DO3A})(\text{CO}_3)]^{2-}$ complexes (see lower Fig. 1).

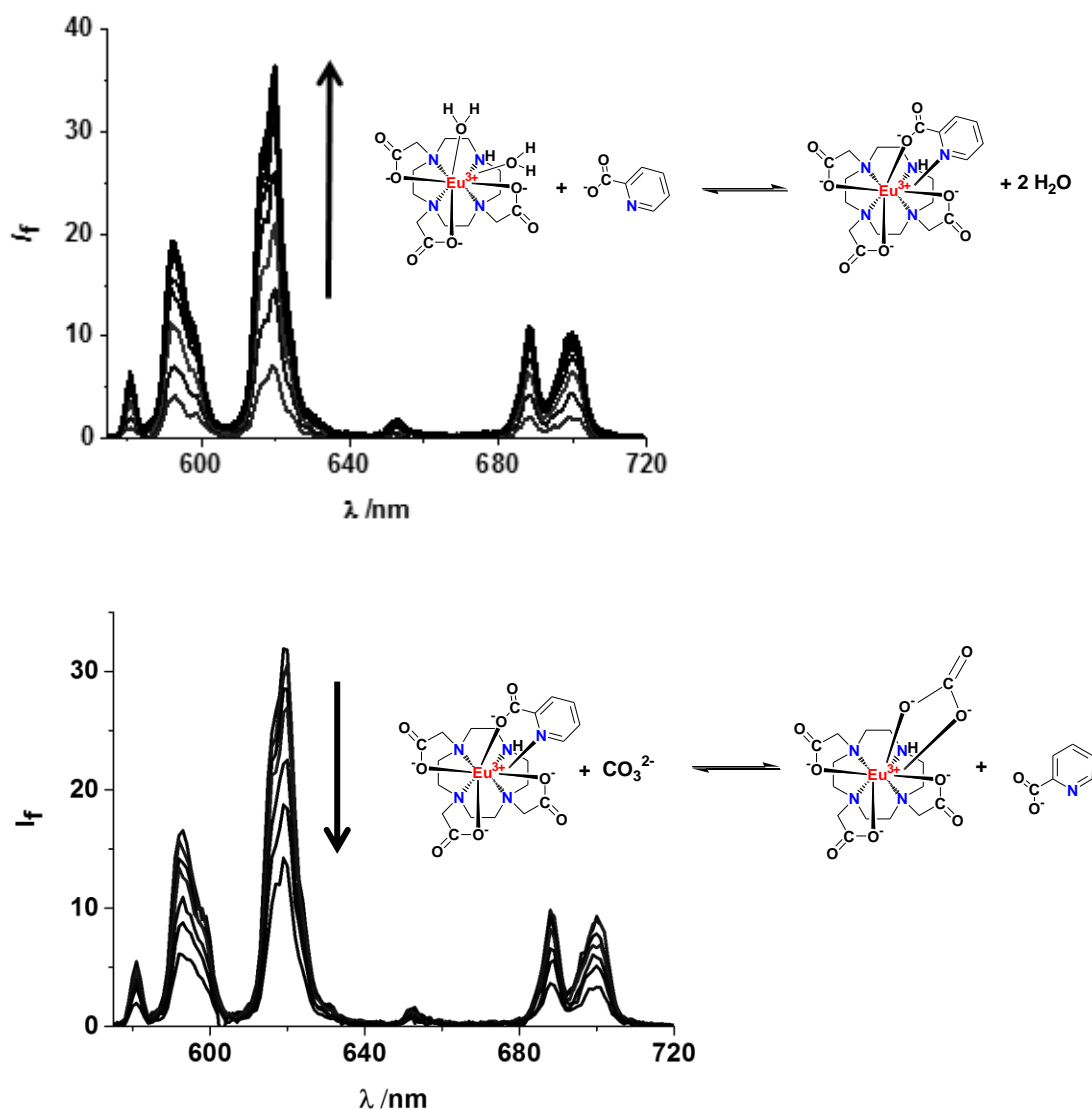


Fig. 1. The emission spectra of $[\text{Eu}(\text{DO3A})]$ complex after addition of picolinate (up) and of $[\text{Eu}(\text{DO3A})(\text{picolinate})]$ complex after addition of bicarbonate.

The decrease of luminescence signal caused by the substitution of the “antenna” ligand with carbonate ions was monitored and thus the bicarbonate concentration in solution can be determined.

4 Conclusions

The bound water molecules in the $[\text{Eu}(\text{H}_2\text{O})_2(\text{DO3A})]/[\text{Eu}(\text{H}_2\text{O})_3(\text{DO2A})]$ complexes undergo substitution with a picolinate-like ligand to form the metal complexes of enhanced luminescence. This picolinate-like ligand then can be substituted with various anions to form more stable ternary adducts. Due to differences in the luminescence and

stability constants of these ternary adducts, the Eu(III) complexes can be used as probes for the detection of various organic substances (picolinic, dipicolinic, or fusaric acid) and anions (bicarbonate, oxalate). This new analytical method is useful for fast and selective determination of substances in environmental analysis.

Acknowledgement

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P63 (SALD) ICP-MS TO EXAMINE THE EFFECT OF COPPER ON WEDELOLACTONE CYTOTOXICITY TOWARDS BREAST CANCER CELLS

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Summary

In this study, the effect of copper on wedelolactone cytotoxicity towards breast cancer cells was examined. To monitor copper loading of cells, SALD ICP-MS (substrate assisted laser desorption inductively coupled plasma mass spectrometry) and conventional nebulizing ICP-MS analysis were used. The SALD technique provided direct and fast analysis of liquid samples in the form of dried droplets with minimal sample preparation and sub-microliter sample volume requirement. Furthermore, the SALD allowed direct analysis of samples in organic solvents, which could not be directly nebulised to ICP-MS. The experiments showed that copper chelation can drastically reduce wedelolactone cytotoxicity.

1 Introduction

1.1 Wedelolactone cytotoxicity effect

The wedelolactone is a plant polyphenolic coumestan isolated from *Eclipta alba* and *Wedelia calendulacea* [1]. Its anti-cancer properties were described by many groups, several proteins and signalling pathways were identified as wedelolactone targets including NFkB, steroid receptors, MAPK kinases and topoisomerase II [3-10]. These effects were documented on a broad range of the cancer cell lines including the breast, colon, hepatocellular, pituitary and neuroblastoma [2-6]. We hypothesize that the wedelolactone cytotoxicity can be enhanced in the presence of copper ions due to the wedelolactone ability to reduce Cu^{2+} to Cu^+ which results in production of reactive oxygen species (ROS) and DNA damage *in vitro*. In this study, the effect of copper wedelolactone cytotoxicity was evaluated.

1.2 SALD

The SALD is a sample introduction technique of liquids to the ICP-MS. This technique is based on deposition of the liquid sample onto a highly absorbing substrate (polyethylene terephthalate plate) and subsequent complete desorption of dried droplets using a pulse UV laser [11]. Main benefits of this technique in comparison with conventional nebulizing ICP-MS technique are minimal sample preparation, consumption of samples in sub-microliter volumes, high sensitivity with LOD in femtogram range, sample plates archiving, direct elemental analysis of organic solvents (e.g. lysed cells in DMSO) and coupling with column separation techniques [12].

2 Experimental

2.1 Sample preparation

Three various sets of cells were prepared to examine the effect of copper on the cytotoxicity of wedelolactone. The first set of cells was pretreated with either permeable (neocuproine) or impermeable (bathocuproine) copper chelator agent and exposed to wedelolactone/DMSO for 48 hours. The second set of cells was treated with copper and wedelolactone either simultaneously for 48 hours or cells were loaded with copper and subsequently treated with wedelolactone. In the last experimental setup, the wedelolactone cytotoxicity was measured after transient transfection either with human copper transporter-expressing vector (hCtr1) or empty vector (cmv) as a control. The wedelolactone cytotoxicity was evaluated by propidium iodide staining of dead cells. Number of propidium iodide positive cells was analysed by a flow cytometry.

2.2 Sample analysis

Raw solutions of lysed cells were applied by a micropipette onto a PET plate as 200 nL droplets. Then the plate with samples was inserted to the ablation cell (model UP213, New Wave Research, Inc., USA) equipped with a 213-nm Nd:YAG pulse laser, 3D positioning system and built-in CCD camera for visual control of ablated samples. The dry aerosol created during the ablation was analysed by the ICP mass spectrometer (Agilent 7500ce ICP-MS, Agilent, USA). During the analysis, ratio of isotopes of ^{63}Cu and ^{65}Cu was monitored for possible polyatomic interferences exclusion.

High volume samples and samples with a high level of copper were analysed using conventional nebulizing ICP-MS. Before the analysis, the samples were diluted with 4% nitric acid (ANAL PURE) to a minimal volume 5 mL.

3 Results and discussion

In the experiment with the first set of cells, the cytotoxicity of wedelolactone was reduced by cell permeable copper chelator neocuproine. Cells simultaneously treated with wedelolactone and copper showed lower propidium iodine staining, indicating significantly reduced wedelolactone cytotoxicity. This was presumably caused by altered ability of wedelolactone-copper complexes to penetrate through cell membranes. To evaluate the effect of increased uptake of copper on wedelolactone cytotoxicity, the cells were first transfected hCtr1-expressing vector or mock-transfected, pretreated with copper and exposed to wedelolactone. For confirmation of impaired hCtr1 function, we analyzed levels of copper by SALD ICP-MS, finding its concentration approximately three times higher compared to mock-transfectants. We observed that cell death induced by wedelolactone is enhanced in copper-pretreated cells overexpressing copper transporter hCtr1. This analysis clearly demonstrated that supplementation of media with copper sulphate resulted in an increased copper intracellular level.

4 Conclusions

In this study, the (SALD) ICP-MS technique was successfully employed to examine the effect of copper on wedelolactone cytotoxicity towards breast cancer cells. The experiments demonstrated that the copper chelation can drastically reduce wedelolactone cytotoxicity and cell death induced by wedelolactone is enhanced in copper-pretreated cells overexpressing hCtr1 gene.

Acknowledgment

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P64 **GLUCOSE OXIDASE IMMOBILIZED AS LENTIKATS
BIOCATALYST FOR INDUSTRIAL APPLICATIONS**

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Summary

Immobilization of enzymes into polyvinyl alcohol (PVA) matrix in the form of Lentikats Biocatalyst (LB) brings several important advantages. Lentikats Biocatalyst possesses excellent physical and mechanical characteristics (high elasticity, abrasion resistant) that provide robustness and long lifetimes. Moreover, PVA matrix is biologically non-degradable, has zero toxicity and is an inexpensive immobilization material without side effects on the biochemical processes. Complexly, Lentikats Biotechnology offers several important benefits, such as relatively simple production procedure, no requirement for pre-treatment of input material and high productivity. Optimization of glucose oxidases (GOXs) immobilization into PVA matrix was done. Oxidation of β -D-glucose to D-glucono- δ -lactone, with the concurrent release of hydrogen peroxide, by free and immobilized GOXs was used for activity determination. Enzymes activity recovery of 81.5 and 86.1 % was reached for immobilized enzymes GOX-I (protein concentration used for immobilization was $2.8 \cdot 10^{-3}$ mg/g_{LB}, 10% LB reaction volume loading) and GOX-II (protein concentration used for immobilization was $9.6 \cdot 10^{-3}$ mg/g_{LB}, 5% LB reaction volume loading). Activity of Lentikats Biocatalyst with immobilized enzyme GOX-I and GOX-II was determined as 0.088 U and 0.094 U/g of Lentikats Biocatalyst, in this order. The immobilized GOXs were stable at least during 10 repeated batch conversions and for min. within 4 weeks at storage conditions (this parameter is still monitored).

1 Introduction

Immobilization of biomaterial (cells or enzymes) is an evolving technology in the field of biocatalysis and is considered a primary strategy to overcome limited stability of biocatalysts [1]. Immobilized biomaterial is currently widely used for variety of potential applications. The immobilized biomaterial can be separated from the reaction mixture and reused. Main benefits in immobilization we can see in prevention the immobilized biomaterial from being exposed to harsh conditions, such as high temperature, surfactants, and oxidizing agents etc. The immobilized biomaterial is widely used in pharmaceutical&food industry, bioremediation, detergent or textile industry and in the others industrial fields [2].

Lentikats Biotechnology is patented technology and it presents a modern solution for immobilization (encapsulation) of cells or enzyme into a porous hydrogel matrix made of polyvinyl alcohol (PVA), as Lentikats Biocatalyst (Fig. 1). A part from its high immobilization capacity, the matrix possesses excellent physical and mechanical characteristics (elasticity, no abrasion), which provides a lasting durability of the Lentikats Biocatalyst. The unique lentil-like shape ensures unlimited substrate diffusion to the immobilized cells or enzyme, while still retaining a convenient size for easy separation of the biocatalyst from the reaction media. Moreover, the PVA matrix is biologically non-degradable and has zero toxicity. It is an inexpensive immobilization material with no side effects on the biochemical process.

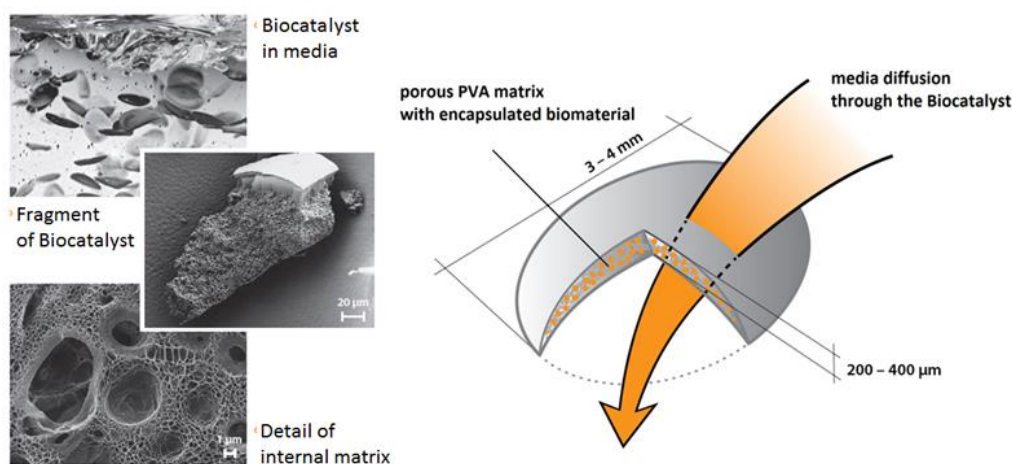


Fig. 1. Lentikats Biocatalyst (in left – Biocatalyst in media, fragment of Biocatalyst and detail of internal PVA matrix, in right – principle of Biocatalyst, biomaterial encapsulated into polyvinyl alcohol (PVA) matrix).

Immobilization into polyvinyl alcohol (PVA) matrix represents a highly effective technique for the immobilization of glucose oxidase (GOX) for commercial application because large-scale production of Lentikats Biocatalyst has been achieved and because this technology has already found utilization in several fields in industry.

2 Experimental

Two glucose oxidases (GOX-I from *Penicillium amagasakiense* and GOX-II from *Aspergillus niger*; provided by DuPont, NLD) were immobilized into a PVA matrix in the form of Lentikats Biocatalyst by standard LentiKat's procedure.

Activity determination of free and immobilized GOXs was performed in volumes 1-10 mL at laboratory temperature in phosphate buffer (pH 7.5). The reaction mixtures contained 2-10% (w/v) loading of Lentikats Biocatalyst with immobilized GOXs, which corresponds to the final concentration of the catalyst in the reaction ($2.8 \cdot 10^{-4}$ for GOX-I and $4.8 \cdot 10^{-4}$ for GOX-II mg/mL). The quantitative formation of a pink complex was measured spectrophotometrically at 515 nm using reversible oxidation of β -D-glucose to D-glucono- δ -lactone (see Fig. 2).

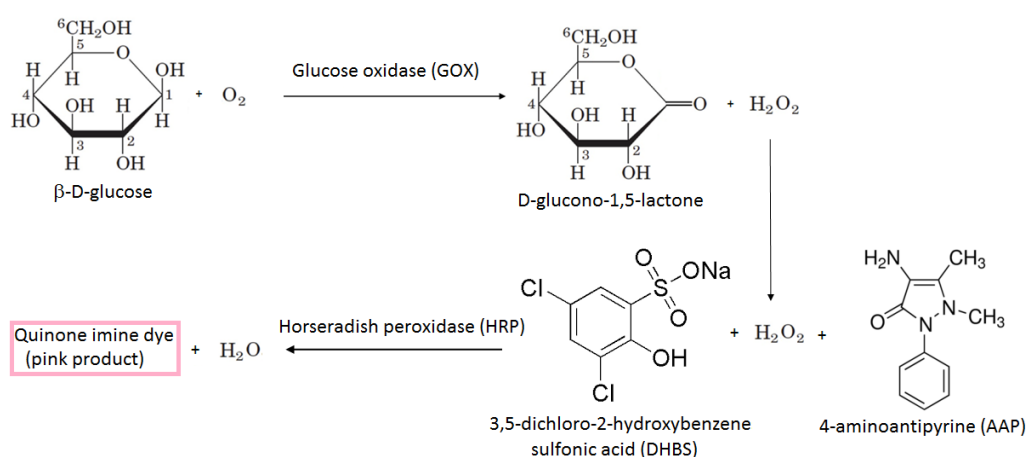


Fig. 2. General reaction scheme of glucose oxidase activity determination.

3 Results and Discussion

For the immobilized enzymes GOX-I and GOX-II was reached 81.5 and 86.1 % of activity recovery (see Tab. 1). Activity of immobilized enzyme GOX-I and GOX-II was determined as 0.088 U and 0.094 U/g of Lentikats Biocatalyst, in this order. The immobilized GOXs were stable at least during 10 repeated batch conversions and for min. within 4 weeks at storage conditions (this parameter is still monitored).

Table 1. Results of the activity determination of immobilized GOX-I and GOX-II.

Glucose oxidase (GOX)	Enzyme	Free	Immobilized (as Lentikats Biocatalyst, LB)		
Reaction volume (mL)	GOX-I/-II	1	10		
LB filling in reaction (%)	GOX-I/-II	-	2	5	10
Concentration of protein in LB ($\text{mg}_{\text{protein}}/\text{g}_{\text{LB}}$)	GOX-I	-	$1.4 \cdot 10^{-2}$	$5.6 \cdot 10^{-3}$	$2.8 \cdot 10^{-3}$
	GOX-II	-	$2.4 \cdot 10^{-2}$	$9.6 \cdot 10^{-3}$	$4.8 \cdot 10^{-3}$
Concentration of protein in reaction ($\text{mg}_{\text{protein}}/\text{mL}$)	GOX-I	$2.8 \cdot 10^{-4}$			
	GOX-II	$4.8 \cdot 10^{-4}$			
Activity ($\text{U}/\text{mg}_{\text{protein}}$)	GOX-I	38.72	22.86	30.00	31.58
	GOX-II	24.17	15.88	20.38	19.51
Activity ($\text{U}/\text{g}_{\text{LB}}$)	GOX-I	-	0.320	0.168	0.088
	GOX-II	-	0.381	0.196	0.094
Activity yield (%)	GOX-I	-	59.0	77.5	81.5
	GOX-II	-	67.1	86.1	82.4

4 Conclusions

Successful immobilization into PVA matrix with high enzyme activity recovery.

High productivity, min. 10 repeated batch conversions.

Long time storage stability, min. 4 weeks (still monitored).

Acknowledgement

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**P65 SEPARATION OF NANOPARTICLES IN THE HUMAN
BLOOD PLASMA BY FLOW FIELD-FLOW FRACTIONATION**

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Summary

The aim of the study was the first attempt to develop and test methodology for qualitative determination of erythropoietin concentration by the use of asymmetric flow field-flow fractionation (A4F) in biological samples (plasma of human blood).

1 Introduction

A4F is a technique that provides fast and smooth separation of all kinds of nanoparticles in a liquid medium. The sample is separated in channel without the stationary phase [1]. Separation of the particles may occur due to non-uniform interactions of the sample components with the external field directed perpendicular to the liquid flow channel [2, 3].

2 Experiment

The blood was stored in tubes with EDTA solution to decrease clotting. The plasma was separated by centrifugation. Erythropoietin was externally added to the samples with the medicine NeoRecormon® 500 UI (Riche). A4F analysis was performed with saline as liquid carrier. Wavelength of UV-Vis detector was set to 240 nm. The flow rate through the channel was 0.5 ml/min while the cross flow was 3.0 ml/min.

The fractions obtained during A4F separation were concentrated prior other analyses. Purification was performed by the use of ultrafilter Amicon Ultra-4 Centrifugal Filter Units Merck Millipore. Such prepared material was analyzed by Direct Detect Near-Infrared Spectrometer. 2ul of each fraction was dried and analyzed at the PTFE membrane to provide the protein concentration in the individual fractions. Moreover Gel Electrophoresis was also performed in a vertical plate polyacrylamide gel. Electrophoresis was carried out in a batch system for about 30 minutes at a voltage of 200 V. After completion of the process, the resulting gel was stained in CVB.

3 Results and Discussion

Comparison A4F fractograms of plasma samples without (Figure 1) and with EPO (Figure 2) shows significant differences. However at this stage of research, it is unclear which of the resulting fractions externally introduced erythropoietin is.

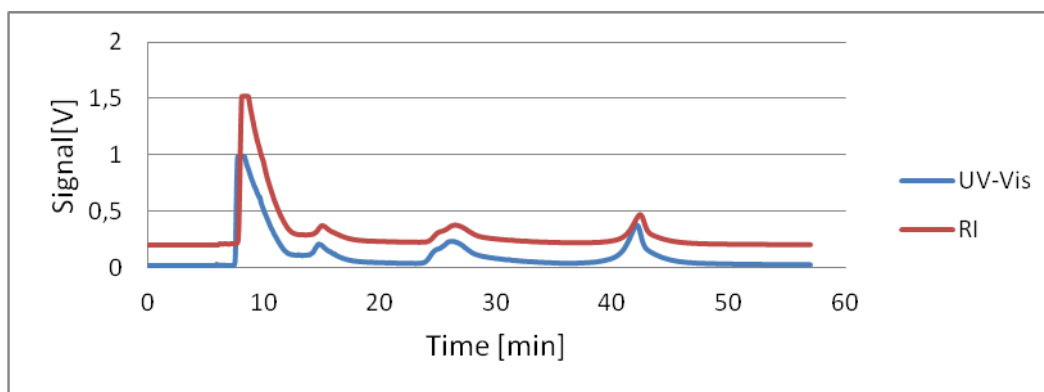


Fig. 1. Fractogram without the addition of erythropoietin in plasma.

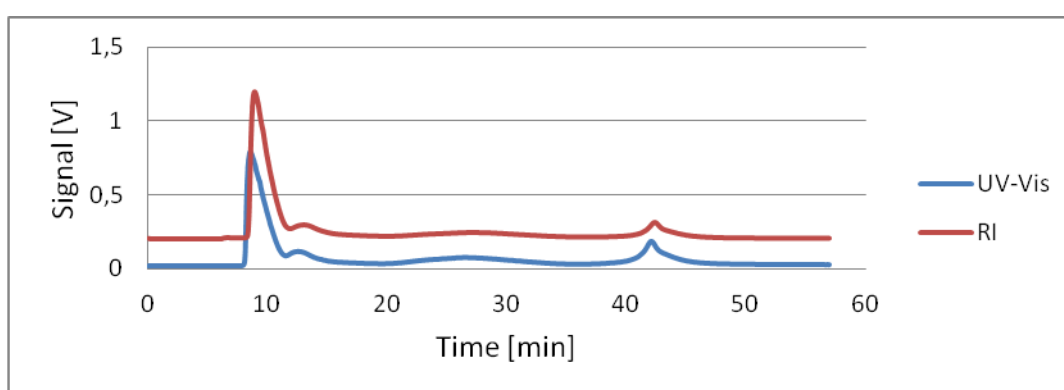


Fig. 2. Fractogram of plasma containing erythropoietin.

Comparing the electrograms in both cases additional bands were observed in two fractions. It were a protein bands corresponding to a weight of 28kDa and the 14kDa for the second and the third fraction, respectively. Externally supplied erythropoietin may be located in the second fraction.

4 Conclusions

The presence of erythropoietin externally supplied to the human blood was detected by A4F. Furthermore, the optimum conditions were found to perform analysis using saline as a carrier liquid. Identification of erythropoietin in particular fraction will be the subject of the next test.

Acknowledgment

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**P66 CHIRAL SEPARATION OF DRUGS USING A NEW
DERIVATE OF VANCOMYCIN IN CAPILLARY
ELECTROPHORESIS**

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Summary

In our work, we developed a new method for chiral separation of cetirizine and other drugs using capillary electrophoresis. The method uses newly synthesized derivate of macrocyclic glycopeptide antibiotic vancomycin as chiral selector (CS). While further optimization of the method is needed the results are very promising. Enantiomers of cetirizine were resolved under 3 minutes using this method.

1 Introduction

Vancomycin is a naturally occurring chiral compound which contains a variety of functionalities that are known to be useful for enantioselective interactions. The newly synthesized compound was prepared by Prof. Armstrong's group by reshaping the well-known structure of vancomycin to contain extra two carboxylic groups. The derivate has several characteristics of vancomycin: absorbs strongly below 250 nm, hydrochloride of the compound is soluble in water; excessive temperatures, pH extremes and "old" solutions can cause stability problems leading to formation of white precipitate [1]. Chiral separation can be optimized by changing one or more of experimental conditions such as the capillary temperature, buffer pH, capillary length, buffer concentration or ionic strength and others.

2 Experimental

All experiments were performed on the capillary electrophoresis HP 3DCE, Agilent Technologies. It was equipped with DAD detector and 50 μm (i.d.) \times 33.5 cm (25 cm to detector) fused silica capillary. Vancomycin solution was introduced into the capillaries using pressure of 50 mbar for 120 sec. Borate buffer solution was prepared by adjusting the pH of 50 mM boric acid solution with 0.1 M sodium hydroxide solution to pH 8.0. Running buffer 50 mM sodium borate pH 8.0 with 0.1 mM CTAB was used.

3 Results and discussion

High absorbance of the new CS did not allow addition of the CS to the run buffer solution. Because of this, partial filling of capillary was chosen as the best alternative. The filling time using pressure of 50 mbar was 30, 60, 120, 150 and 180 sec. Unfortunately the CS at pH 8.0 showed low migration times (as result of combination of high mobility of EOF and ionization of carboxylic groups) and overlapped the signal of cetirizine. Adjusting of EOF was needed. Addition of CTAB was used which resulted in lowering the mobility of EOF. This allowed reversal of migration of the highly absorbing vancomycin zone from negatively to positively charged electrode. Without the disturbance caused by the CS the chiral separation of cetirizine enantiomers as model compounds can be recorded within 3 minutes.

4 Conclusions

A new method for chiral separation of cetirizine and other drugs using capillary electrophoresis was developed. This method presents an approach for usage of the new derivate of vancomycin. To further optimize the method addition of nanoparticles will be used.

Acknowledgements

The financial support of the research by the Ministry of Education, Youth, and Sports of the Czech Republic (NPU LO 1305) is gratefully acknowledged. P.S. thanks the Endowment Fund of Palacký University for her support.

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P67 NOVEL IMAGING METHOD FOR WIDE CONCENTRATION RANGE SAMPLE ANALYSIS IN A SINGLE INJECTION WITH CE

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1 Introduction

One of the most efficient and high-throughput bioseparation methods for therapeutic antibody N-glycan analysis is capillary electrophoresis (CE). The capability to analyze even very low sample amounts made CE an attractive bioanalytical technique. Most of

the commercially available capillary electrophoresis systems are using single point detection approaches on the capillary such as photodiode, PMT, laser, etc. This limits the ways of data acquisition to one dimension, thus the results of the data evaluation process. We developed a novel, image analysis based, LED induced fluorescence detection system with custom filter algorithms - that is different from the whole-column fluorescence-imaged capillary electrophoresis approach.

2 Experimental

The system consists of a confocal optical setup, a minicomputer for injection/separation control including a Charge-Coupled Device attached to the optics, and an image analyzing-and-driver software package. The incoming color images are stored and processed on the fly by channel filter and image calculator operations resulting in mean pixel intensity value plots (electropherograms).

3 Results and Discussion

The suggested imaging technique allows the evaluation of data collected from the injection of highly concentrated or very diluted sample by virtually adjusting the detection window parameters on the capillary with filtering algorithms, thereby modifying the intensity-range of the signal without the necessity of reinjecting the sample.

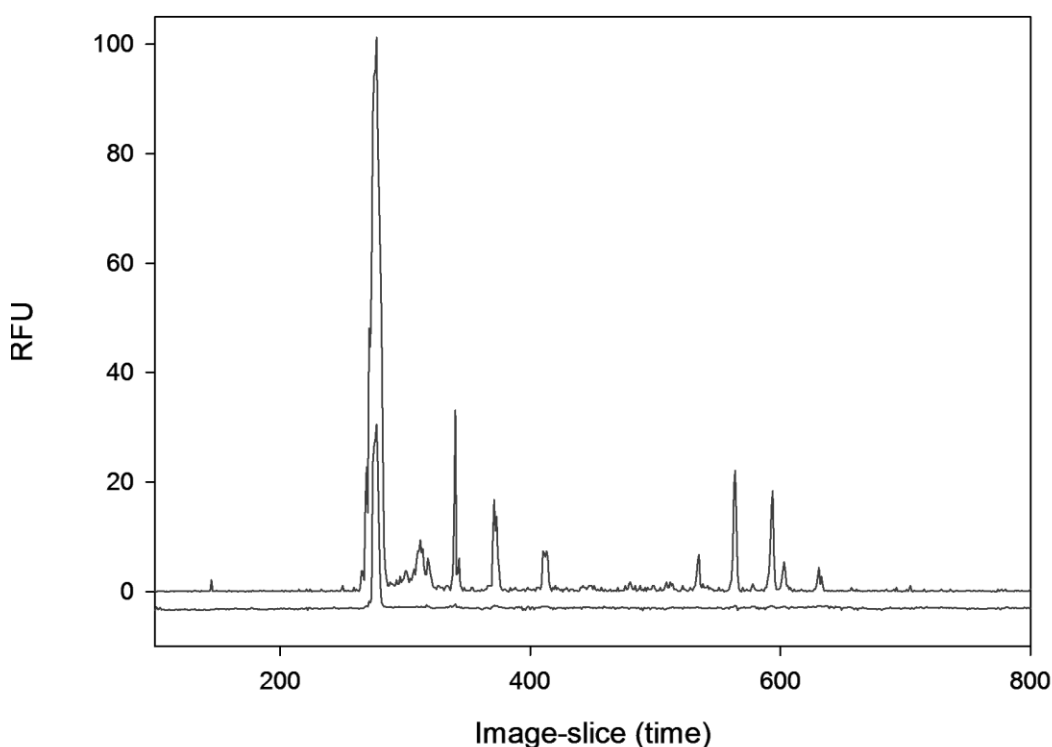


Fig.

1. Separation of IgG N-glycans from low concentration (2 mg/ml) sample. Lower trace: Original separation. Upper trace: Virtual separation of the same sample with adjusted window level parameters.

4 Conclusions

Recently developed high sensitivity CCD/CMOS detectors can be readily implemented in CE-LEDIF to increase the resolution by applying different image-analyzing techniques. This includes but not limited to intensity, segmentation or other optical characteristics based property, combined with separation resolution enhancer fluorescent dyes emitting at different wavelengths, e.g., turquoise dye, TEAL, APTS, etc. Storing all collected raw data offers the option for re-processability of the images. It largely contributes to the on-demand refinement of peak intensity by introducing a two-dimensional (array based) detection instead of a one point like measuring technique.

Acknowledgement

The authors gratefully acknowledge the support of the Momentum (Lendulet) grant # 97101 of the Hungarian Academy of Sciences (MTA-PE Translational Glycomics Group) and the NKFIH (K 116263) grant of the Hungarian Government. The stimulating discussions with Dr Frantisek Foret, is also greatly appreciated.

P68 THREE INTERNAL STANDARD BASED INSTANT GLYCAN STRUCTURE ASSIGNMENT METHOD

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Summary

Despite the ever growing use of capillary electrophoresis in biomedical research and the biopharmaceutical industry, the development of simplified data interpretation methods is falling behind. In this presentation we report on the design and the implementation of a triple-internal standard method that would alleviate the need of maltooligosaccharide ladder runs for glucose unit (GU) calculation. With this novel utilization of the well-chosen three internal standards of maltose, maltotriose and maltopentadecaose, the method presented here provides accurate and reliable results even with data collected at a range of conditions (e.g. various capillary lengths, temperatures, coatings, separation voltages, and injection methods).

1 Introduction

Glucose unit (GU) calculation plays an important role in glycan identification. Despite its well-established role in the chromatography field, CE based data processing and interpretation are still in its early stages. But, the knowledge of the migration behavior of glycans in CE separations allows precise prediction of a virtual ladder based on measured standard migration times, which enables calculation of the GU values without using maltooligosaccharide ladder runs.

2 Experimental

N-CHO Carbohydrate Analysis Kit (Sciex, Brea, CA) was used for sample preparation and analysis. Bare fused silica (BFS) capillaries were from Polymicro Technologies (Phoenix, AZ). Human Immunoglobulin G (IgG) and all other chemicals were purchased from Sigma Aldrich (St. Louis, MO). Capillary length and type (BFS and NCHO), separation temperature and separation voltage were altered. An inter-instrument study was also implemented using PA 800 Plus and P/ACE MDQ systems.

3 Results and Discussion

By the utilization of the inner and co-injected standards, a virtual ladder was accurately established and used for instant glycan structure assignment via GU unit calculation (Figure 1). The calculation method was tested on the major N-linked carbohydrates released from human IgG separated by different capillary length, type and conditions (temperature, separation voltage, injection method).

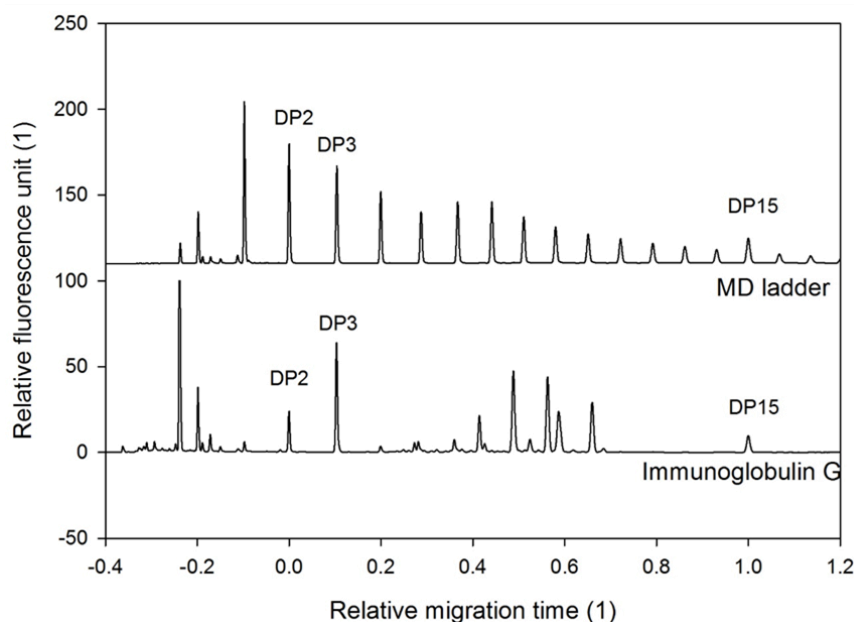


Fig. 1. Capillary electrophoresis separations of the APTS-labeled human Immunoglobulin G N-glycans (lower trace) and an accompanying linear maltooligosaccharide ladder (upper trace) including the three suggested internal standards of DP2, DP3 and DP15.

It was found that the three internal standard based glycan structure assignment method provided accurate peak identification without running the maltooligosaccharide ladder. The highest calculated relative standard deviation was 1.07%, which is well below the reported deviation values of other databases [1].

Acknowledgement

This work was supported by the MTA-PE Translational Glycomics program (#97101) and the NKFIH K116263 grant of the Hungarian Government.

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P69 COMBINATION OF X-RAY MICRO-COMPUTED TOMOGRAPHY AND LASER-ABLATION BASED ANALYTICAL TECHNIQUES FOR 3D HIGH RESOLUTION ELEMENTAL MAPPING

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Summary

Detection and chemical 3D mapping of nanoparticles (NPs) distribution in biospecimen is a challenging task that can be met by simultaneous utilization of imaging techniques. Here we focus on the synergy of X-Ray micro-Computed Tomography (μ CT), Laser-Induced Breakdown Spectroscopy (LIBS) and Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) for investigation of various nanoparticles (Gd, Ti, Pb, etc.) distributed in organs.

Special emphasis is given to the analysis of nanoparticles in a murine kidney in 3D. Firstly, we have demonstrated the 3D visualization capabilities in soft tissues by iodine staining with μ CT. Next, subsequent LIBS and LA-ICP-MS analysis enabled to identify nanoparticles distribution in the sample. Thus, this combination provides a novel analytical approach how to quickly visualize and recognize different elements in soft tissues.

1 Introduction

Multifunctional nanomaterials have been intensively studied in the past two decades in the area of biomedicine for drug delivery and clinical research [1]. Because of heterogenous nature and potential applications of these nanoparticles, a characterization

of the biological distribution of these nanoparticles in tissues and organs is a crucial step in preclinical evaluation.

2 Experimental

μ CT technique is a powerful, non-destructive method capable of visualizing the sample structure before the laser-ablation-based chemical mapping [2]. LIBS is an established analytical technique that is based on spectroscopic analysis of radiation emitted by a micro-plasma induced on the analyte surface by a focused laser pulse. The principle of LA-ICP-MS is based on ablating of sample with a pulsed laser beam and introducing of ions to the mass analyzer.

Using μ CT, LIBS and LA-ICP-MS, the nanoparticles in the sample of mouse kidney were measured, which were injected into the mouse under anesthesia and kidneys were then sampled at certain time after injection.

3 Results and Discussion

Firstly, 3D visualization capabilities in soft tissues has been demonstrated by iodine staining with μ CT (Fig. 1). Voxel resolution of obtained CT data was 2.5 μ m.

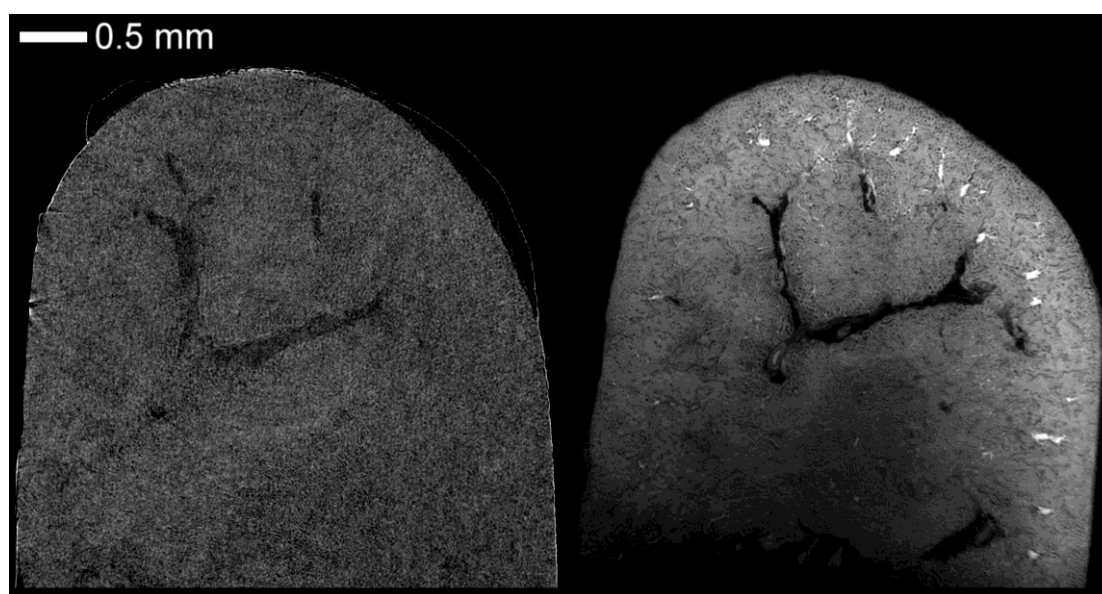


Fig. 1. Tomographic cross-section of non-stained kidney (left) with kidney that was stained by iodine (right).

Next, for specifying high-absorbing places in the kidney, laser-ablation based techniques were used (LIBS and LA-ICP-MS). The map of distribution of gadolinium nanoparticles correlates with CT data and high-absorbing areas in tomographic cross-section. It seems that periphery of the kidney absorbs gadolinium nanoparticles (Fig. 2).

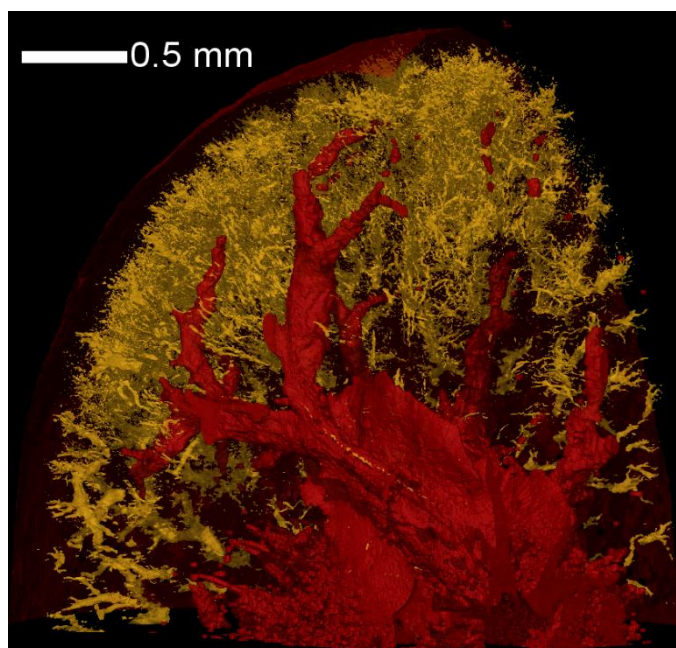


Fig. 2. Detail of 3D visualization of the kidney. Yellow parts represent high absorbing places in the sample bulk.

4 Conclusions

The combination of μ CT with LIBS or LA-ICP-MS brings new possibilities for mapping distribution of nanoparticles in biological samples. Using μ CT, 3D volume is relatively quickly taken, without the need of slicing and destruction of the sample. In one measurement the distribution of heavy metals can be visualized. Complementary laser-ablation techniques can be used to identify chemical composition of the sample.

Here we have demonstrated this approach by mapping gadolinium and titanium nanoparticles in murine kidney.

Acknowledgement

This research has been financially supported by the Ministry of Education, Youth and Sports of the Czech Republic under the project CEITEC 2020 (LQ1601).

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**P70 3D PRINTED ELECTROCHEMICAL CHIP FOR CADMIUM
IONS ANALYSIS IN ZEA MAYS SEEDLINGS - THE MARSELECTRO
PROJECT**

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Summary

This work was focused to study of cadmium ions in *Zea Mays* by 3D printed electrochemical chip. It was revealed that the damage in the *Z. mays* seedlings increased with increase in the concentrations of Cd and the exposition period.

1 Introduction

Colonization of other planets in the solar system is currently a big challenge. The main reason for colonizing other planets will probably be the almost impossible recovery of the nearly depleted number of material resources on the Earth and the dramatic population growth, contributing to significant climatic changes. Terraforming of Mars and its consequent settlement are expected in a not so distant horizon with regard to current conditions on planet Earth. Mars is being studied intensively (e.g., Mission Mars Exploration Rovers – Sojourner, Spirit and Opportunity, Phoenix, Curiosity, Mangaljan probe). At present, it is also expected that the first people will land on Mars between 2014 and 2070 [1]. Owing to the simplicity of electrochemistry, with its sufficient sensitivity, availability and use of instrumentations, there are great possibilities to miniaturize electrochemical devices (e.g., microfluidic Lab-on-a-Chip device). Notably, the sensitivity of electrochemical analysis cannot be affected by miniaturization [2-5]. Therefore, there are immense possibilities to design a portable device as small as a chip [6]. Employing an electrochemical detection system in chip, the proposed project, MARSELECTRO, aims to determine the changes in the concentrations of cadmium (Cd) ions in biological samples.

2 Experimental

Cadmium (Cd) and all other chemicals were purchased from the Sigma-Aldrich. The seedlings of maize (*Zea mays* L.) were kept under dark conditions at 25°C, and were supplemented in triplicate with Cd ions (0, 5, 25, 50, 100 µM). At 24, 48, and 72 h of exposure, the seedlings were harvested and carefully rinsed with distilled water and 0.1 M EDTA. After each time point of the experiment, samples (100 µL) were put into the electrochemical device. Using acrylonitrile-butadiene-styrene, the chip was made on the Profi 3D maker (3DFactories, Czech Republic). *Z. mays* samples (roots or shoots) were homogenised and the Cd ions concentration was determined in the *Z. mays* samples (roots or shoots) by a 3D printed electrochemical chip. Measurements of the electrochemical cells were done in the volume of 1.0 ml; where the electrolyte was 0.2 M acetate buffer (pH 5). Parameters of voltammetry were as follows: initial potential -1.2 V; end potential -0.3 V; potential step 3 mV; and potential of deposition -1.0 V. The designing of the model was carried out using the software Solidworks. Electrochemical measurements were performed using minicomputer-connected potentiostat 910 PSTATmini (Metrohm, Switzerland).

3 Results and Discussion

The fundamental step in the development of the electrochemical platform was the construction of a 3D printed electrochemical cell and the selection of suitable electrodes for long-term measurements. The influence of Cd ions on the growing *Z. mays* seedlings was analysed during the whole experimental period. Germination of the *Z. mays* kernels was more than 90% under control (no Cd) condition; where, the decrease in the germination reached up to 60% with Cd-exposures. It was revealed that the damage in the *Z. mays* seedlings increased with increase in the concentrations of Cd and the exposition period. The obtained calibration curve was strictly linear, within a concentration range from 1.0 to 500 µM with a regression equation $y = 0.6419x - 1.132$ and $R^2 = 0.997$. In *Z. mays* roots and shoots, Cd ion concentrations varied between 1.0 to 20 µM and 0.5 to 8 µM, respectively. Moreover, the levels of total thiols and proteins, antioxidant activity increased with increasing concentration of Cd ions. Premature decline was observed in rhizodermis. The changes in the deposition of lipophilic polymers in root structures and formation of intercellular spaces were also evidenced.

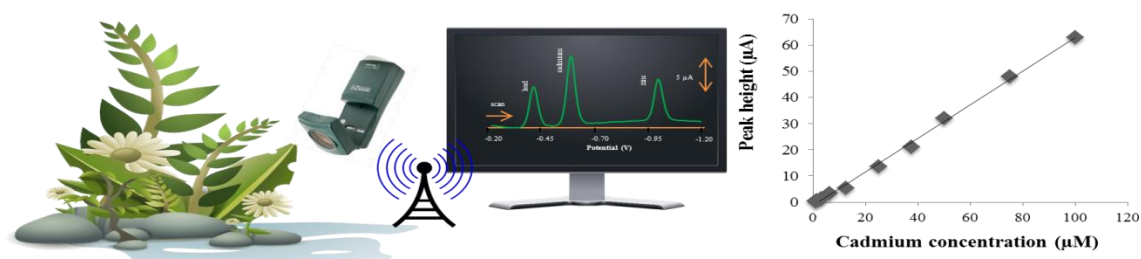


Fig. 1. Scheme of the 3D printed electrochemical chip designed to analyze biological samples. All of the data obtained were transferred to a processing unit. Typical DP voltammogram of Pb(II), Cd(II), and Zn(II) ions (10 µM) can be seen on the screen. The peak height depended on the Cd ion concentrations (0.75–100 µM).

4 Conclusions

Experimentations in this study under the MARSELECTRO project unveiled the possibility of using 3D printed electrochemical chips in the analyses and potential mechanisms of Cd ion-effects on *Z. mays*, used herein as a model plant system. Outcomes can be useful for the experiments to be performed in the Mars Desert Research Station (Utah) in 2017.

Acknowledgement

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P71 DETERMINATION OF ANTIPYRETICS IN PHARMACEUTICALS BY MICROCHIP ELECTROPHORESIS

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Summary

The separation and determination of ibuprofen, diclofenac and acetylsalicylic acid in pharmaceutical preparations by microchip electrophoresis was employed in this work. An on-line combination of isotachopheresis with capillary zone electrophoresis (ITP-CZE) performed on a polymethylmethacrylate microchip with coupled separation channels and conductivity detection was used for this purpose. Optimal ITP separation conditions were achieved with a leading electrolyte at pH 6.5. CZE separations were carried out at pH 7.0 using TES buffer. The developed method was used for the

determination of studied antipyretics with recoveries ranged from 90 to 110% in eleven pharmaceutical samples.

1 Introduction

Antipyretics are substances that reduce fever [1]. They cause the hypothalamus to override a prostaglandin-induced increase in temperature. Most antipyretic medications have also other purposes. For example, ibuprofen (Ibu), diclofenac (Dic) and acetylsalicylic acid (Asp) are non-steroidal anti-inflammatory drugs used primarily as analgesics (pain relievers) with antipyretic properties.

Capillary electrophoresis (CE) is a commonly used analytical technique for determination of Ibu, Dic and Asp in pharmaceutical products [2-4]. Its miniaturized format, microchip electrophoresis (MCE), was widely used in recent years for drug monitoring, although it has not been used for the analysis of antipyretic substances. The on-line combination of isotachopheresis (ITP) and capillary zone electrophoresis (CZE) in a column coupling (CC) configuration of the separation channels is a very effective tool for increasing the separation capacity and sensitivity of CZE.

The aim of this work was to develop the ITP-CZE method for fast and sensitive determination of Ibu, Dic and Asp in commercially available pharmaceutical preparations on the CC microchip with conductivity detection.

2 Experimental

2.1 Instrumentation

ITP-CZE separations were carried out on the polymethylmethacrylate (PMMA) CC microchip with conductivity detection (IonChipTM 3.0, Merck, Darmstadt, Germany). MCE analyzer consisted of electrolyte and electronic unit. MicroCE Win software, version 2.4 was used for monitoring the analysis as well as for collecting the data from conductivity detectors and their evaluation.

2.2 Chemicals and samples

Chemicals used for the preparation of electrolyte solutions and stock solution of model samples were obtained from Sigma-Aldrich (Steinheim, Germany) and Fluka (Buchs, Switzerland). Samples of pharmaceutical preparations (Aspirin, Acylpyrin, Acylcoffin, Acifein, Migralgin, Veral, Glimbax, Nurofen, Nurofen instant, Ibutabs and Brufen) were purchased in local pharmacy. Sample pretreatment included only centrifugation and appropriate dilution.

3 Results and Discussion

ITP separation working conditions (leading electrolyte at pH 6.5 and terminating electrolyte at pH 7.4 with TES as a terminating anion) allowed low-dispersive transfer of analytes into the CZE separation stage. α -cyclodextrin added to the carrier electrolyte (pH 7.0; TES used as a carrier ion) improves the resolution of Ibu and Dic. A typical electropherogram from ITP-CZE separation obtained under optimized conditions is shown in Fig. 1.

Employed working conditions (suppressed electroosmotic and hydrodynamic flow) attained a high reproducibility of migration times and accurate determination of trace concentrations of analytes in model samples. Intra-day repeatabilities of quantitative and qualitative parameters in model samples were calculated from three repeated ITP-CZE runs at three different concentration levels. RSD for peak areas were in the range 0.2–7.6% and RSD values of migration times were within 1.3%.

Microchip with a 900 nL injected volume enabled to reach low concentration limit of detection for the studied analytes in the range from 0.55 to 1.45 mg/L.

Eleven samples of pharmaceutical preparations were analyzed for a content of Ibu, Dic and Asp by proposed ITP-CZE method on the CC microchip. Centrifugation and appropriate dilution of the analyzed samples were the only sample pretreatment steps. Recoveries of studied antipyretics in real samples were in range from 90 to 110%.

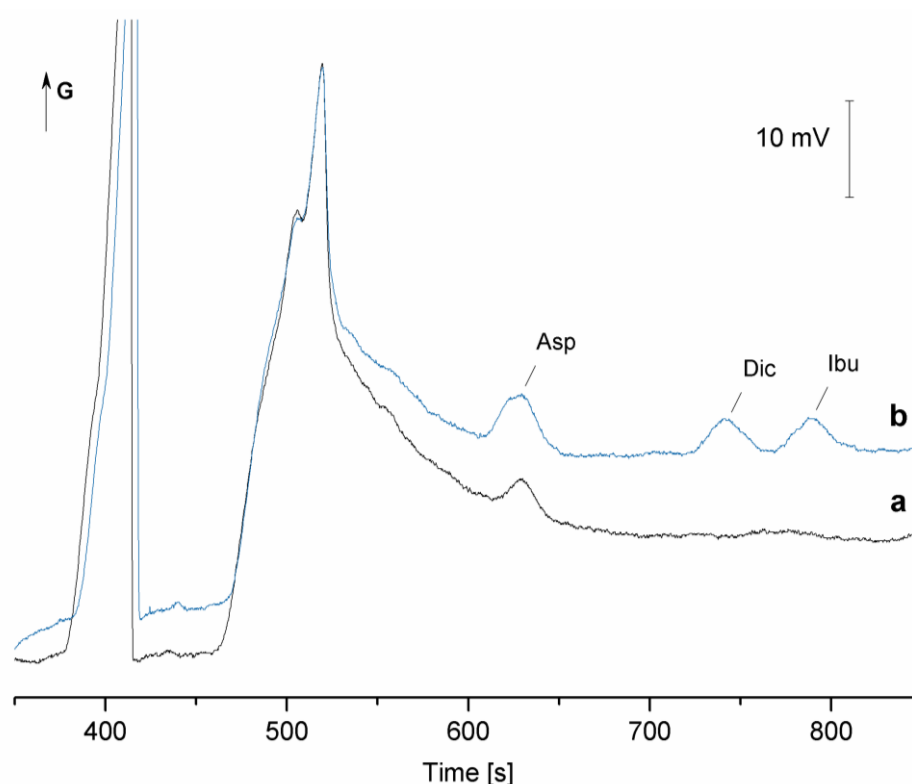


Fig. 1. Electropherograms from the ITP-CZE separation of Asp, Dic and Ibu on the PMMA microchip with conductivity detection. Injected sample: (a) 10 % terminating electrolyte (a blank run), (b) 2.5 mg/L Asp, 10 mg/L Dic and 10 mg/L Ibu in 10 % terminating electrolyte. G = conductivity.

4 Conclusions

This work was focused on a development of MCE method for determination of Ibu, Dic and Asp in pharmaceutical products, which are important for their antipyretic effect. The proposed ITP-CZE method performed on the CC microchip with conductivity detection allowed fast and sensitive determination of studied antipyretics in eleven pharmaceuticals without complicated and time-consuming pretreatment.

Acknowledgement

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P72 CE-nESI/MS IN ELECTRODE-FREE DESIGN: NARROWING THE SEPARATION CHANNEL TO 5 μm

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Summary

Capillary electrophoresis-nanospray/mass spectrometry (CE-nESI/MS) in electrode-free arrangement represents a very simple instrumentation for analysis of biomolecules. So far, CE-nESI/MS analyses in this design were limited to 10-75 μm ID of the separation channel. Although the work with narrower dimensions brings several challenges, there is a great potential for better sensitivity, improved separation power and lower sample consumption. This work is devoted to some practical aspects of experiments in narrow bore channels and systematic evaluation of CE-nESI/MS analyses conducted in capillaries of 25, 15 and 5 μm ID.

1 Introduction

Electrode-free arrangement is the simplest instrumentation for CE-ESI/MS experiments [1]. The operation is based on application of voltage only at the injection end of the capillary, with the opposite end sharpened directly into a nanospray tip. Since a potential drop develops along the capillary due to the capillary resistance and electrospray current, the CE separation occurs. Ideally, the largest part of the applied voltage will span across the capillary with only residual portion used at the tip used for generation of the electrospray plume. Previously published works proved benefits of narrow bore channels for CE-nESI/MS analyses [2, 3]. However, due to several practical challenges (e.g. high pressure, capillary lifetime and worse repeatability due to surface chemistry) the experiments were so far limited to ID $\geq 10 \mu\text{m}$. Since the use

of narrower capillaries should result in improved ionization efficiency, separation power and lower sample consumption, we decided to investigate the main characteristics of 5 μm ID channels. A mixture of seven amino acids was chosen as a model sample.

2 Experimental

The CE separation of mixture of seven amino acids purchased from Sigma Aldrich was conducted in a bare fused silica capillary of 40 cm length and 25, 15 or 5 μm ID with the nanospray tip at one end. The tip was positioned in front of the MS sampling orifice (LTQ, Velos Pro, Thermo Fisher) without any additional electrical connection. The opposite (injection) capillary end was placed in a vial inside a pressurized chamber allowing control of the flow rate in the capillary as well as sample injection - see Fig. 1. The CE separation current was delivered via a Pt electrode inserted into the vial. Water solution of 1% HCOOH (v/v), $\text{pH} = 2.2$ was used as BGE. After the sample injection, high voltage (20 kV) was applied under no flow conditions. In the moment when the fastest analyte reached the tip, the voltage was adjusted to nanospray friendly level and the BGE flow inside the separation capillary was initiated by pressurizing the electrode reservoir. All the experiments were conducted in a clean laboratory.

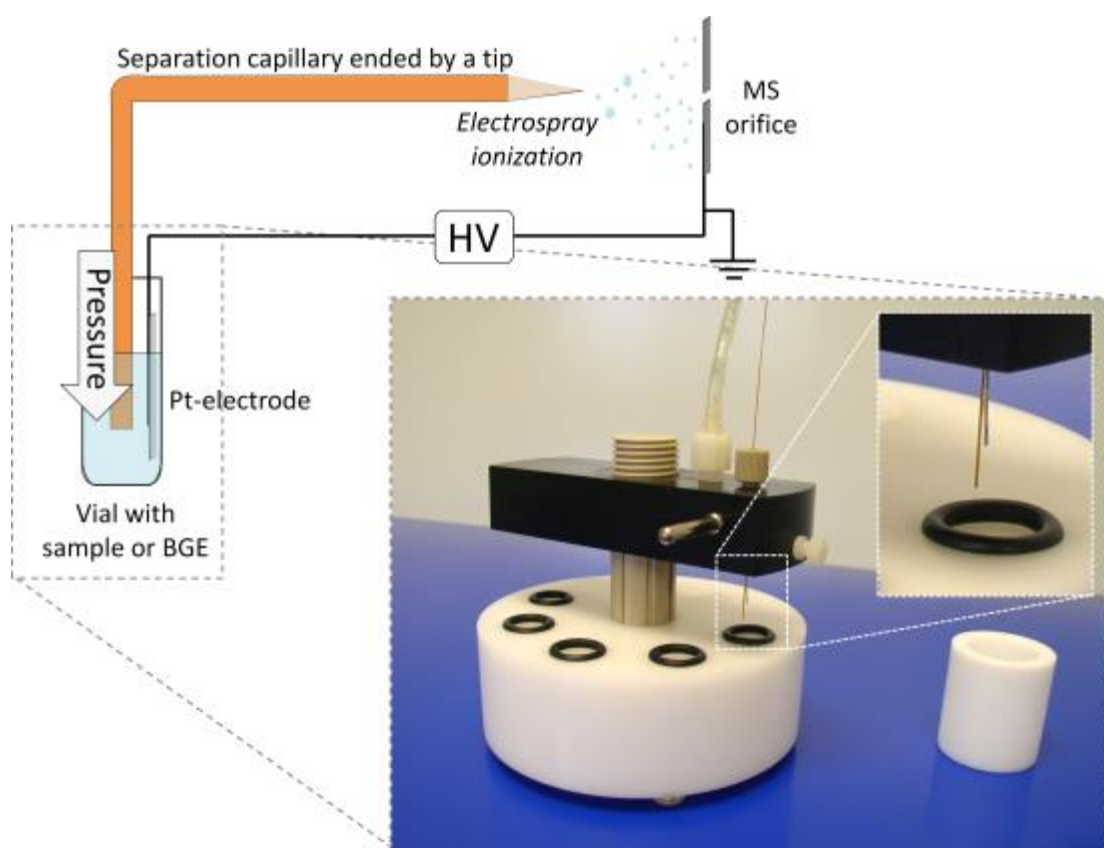


Fig. 1. Instrumentation for CE-nESI/MS in electrode-free arrangement.

3 Results and Discussion

Due to improved ionization efficiency and better robustness resulting from lower flow rate, there is currently increased interest in use of nanospray emitters of very narrow inner diameter. To investigate this effect, the signal of tyrosine was monitored during MS infusion from 25, 15 and 5 μm ID emitters. The signal was observed at a flow rate chosen during preliminary experiments with voltages ranging from 1.5 to 5 kV. The results summarized in Fig. 2 clearly show improved sensitivity for narrower emitter across the tested voltage range.

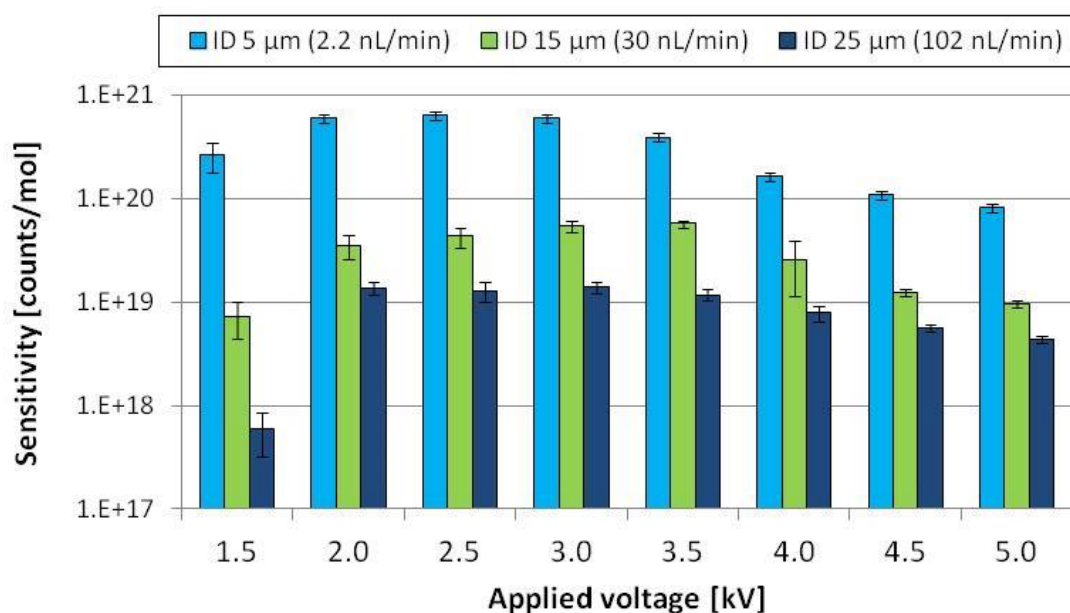


Fig. 2. Sensitivity of the MS analysis during tyrosine ($1 \mu\text{g/mL}$) infusion.. The data are shown in logarithmic scale.

Besides positive influence on ionization during the MS infusion, the narrow bore channels are promising also in terms of separation efficiency. Effective decrease of Joule heating and hydrodynamic profile of the flow to the band broadening has positive influence on the resolution of separation. However, narrow bore capillaries significantly influence the volume to surface ratio leading to stronger surface chemistry effects. Under improperly chosen conditions, sorption can significantly influence efficiency of the separation.

The mixture of seven aminoacids was analyzed by CE-nESI/MS with electrode-free design in capillaries of different inner diameter (25, 15 and 5 μm) under conditions specified in experimental section. Fig. 3 demonstrates improvement of separation power in narrow bore channels. Although, 5 μm ID capillary did not provide ideally shaped peaks, the record was significantly improved after ammonium cations addition taking benefits from the concentration effect of transient isotachophoresis.

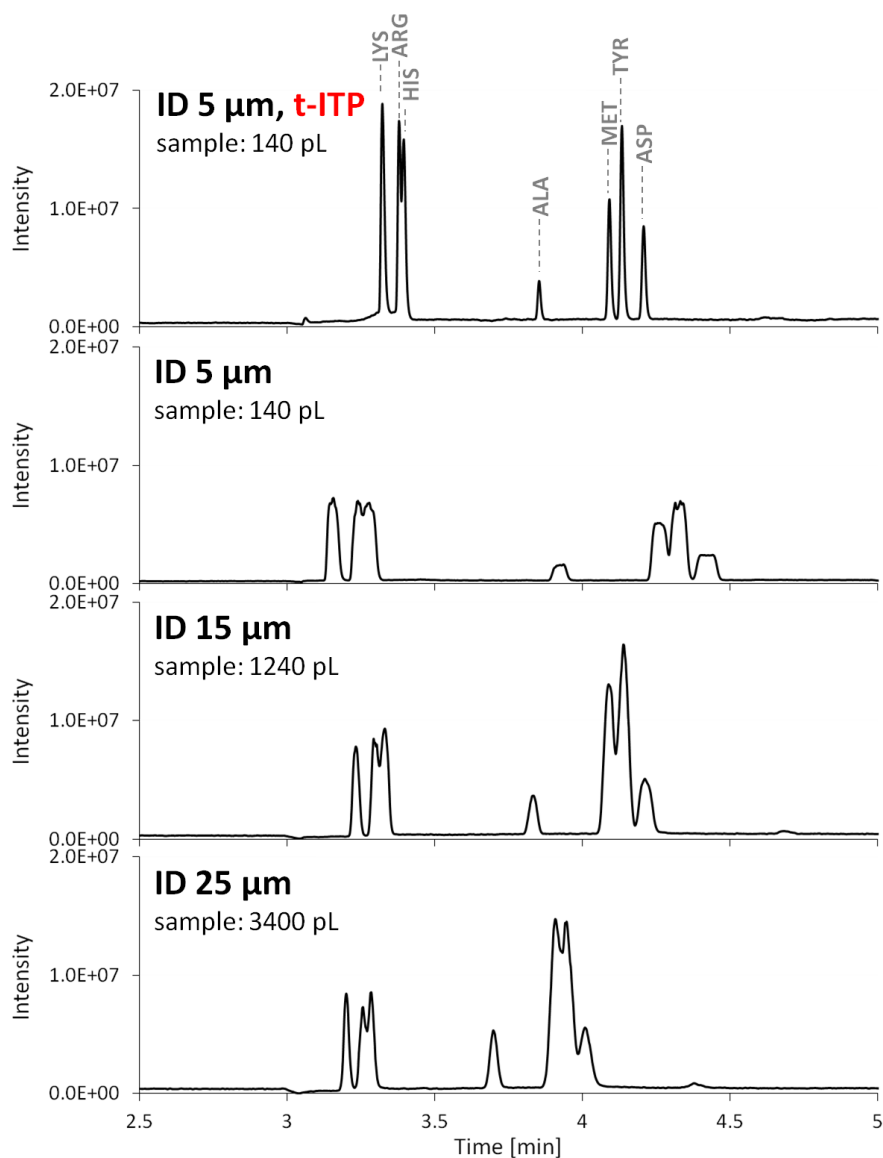


Fig. 3. CE-nESI/MS analysis of seven aminoacids (2 $\mu\text{g/mL}$) in capillaries of 25, 15 and 5 μm ID. Volume of the sample in the graphs stands for its consumption.

4 Conclusions

In this work, a systematic evaluation of capillary performance of 25, 15 and 5 μm ID for CE-nESI/MS in electrode-free arrangement was presented. The positive influence on sensitivity and separation resolution was proved.

Acknowledgement

The work has been supported by the Czech Science Foundation (P206/12/G014, GA15-15479S) and Institutional support RVO: 68081715. The authors wish to thank Jan Prikryl for the help with device construction.

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P73 MICROFLUIDIC DEVICE FOR CELL COUNTING AND CHARACTERIZATION

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Summary

Integration of cell counting unit is an important step in developing of microfluidic device for cellular analysis. Here we present a microfluidic device for cell counting based on resistive pulse sensing.

1 Introduction

Microfluidic devices show great potential for analysis of cell populations as a single microfluidic chip can be designed to perform multiple operations, e.g. lysis and staining, allowing to analyze injected cells in small groups or one by one. In such experiment it is important to precisely control the number of entering cells. Therefore integration of a cell counting unit represents an important step in development of microfluidic device for cellular analysis.

Cells flowing through the microchannel can be registered visually using camera and software image analysis. This approach requires a fast camera with objective and software algorithm to recognize cell moving through the microchannel, distinguish it from the background and other cells in case of multiple cells moving in a group.

Resistive pulse sensing is an alternative approach that is based on sensing disruptions in current flow caused by cells passing through the narrow section, often called the sensing gate. The greatest electric current density is at the sensing gate where volume of conductive electrolyte is the smallest thus the overall resistance of a microchannel is given by this narrowest section. When a non-conductive particle enters this section conductivity drops proportionally to the volume of the particle. This phenomenon also allows the system to distinguish a particle size according to the height of resistive pulse. In terms of microfluidic cellular analysis this ability could be used as the first characterization of cells or as a sorting criterion.

2 Experimental

2.1 Microfabrication procedure

Design of microfluidic device was created in AutoCAD (AutoDesk, USA). At first a replication mask was created by exposing this design using a high-resolution direct laser-writing system μ PG 101 Micro-pattern Generator (Heidelberg Instruments, Germany) on commercially available photomask blank substrates (Nanofilm, USA). This replication mask was then used to transfer the design on borosilicate glass wafers coated with a 50 nm layer of chromium and a 50 nm layer of gold as masking metals and S1805 positive photoresist (Dow Electronic Materials, USA). These masking metal layers were sputtered using a sputter-coater Q300TD (Quorum, England). After development of photoresist and etching of masking metal layers the borosilicate wafer was etched in a diluted mixture of sulfuric and hydrofluoric acid at 45°C for 11 minutes to reach a depth of 15 μ m. Inlet holes were drilled in the cover plate which was then thermally bonded to the microchannel plate in a muffle furnace at 620°C for 10 hours.

The plastic frame containing platinum electrodes and reservoirs for cell suspension, buffers and reagents was designed in SketchUp (Google, USA) and printed out using a fused deposition modelling based 3D printer Easy3DMaker (3D Factories, Germany).

2.2 Resistive pulse sensing

The microfluidic device was placed on a SVM 340 synchronized video microscope (LabSmith, USA) to have a visual control over cells running through the sensing gate. Colibrick A/D converter and Clarity Chromatography Data Station (Data Apex, Czech Republic) were used to monitor resistive pulses by measuring voltage as depicted in representation of sensing circuitry in Fig.1.

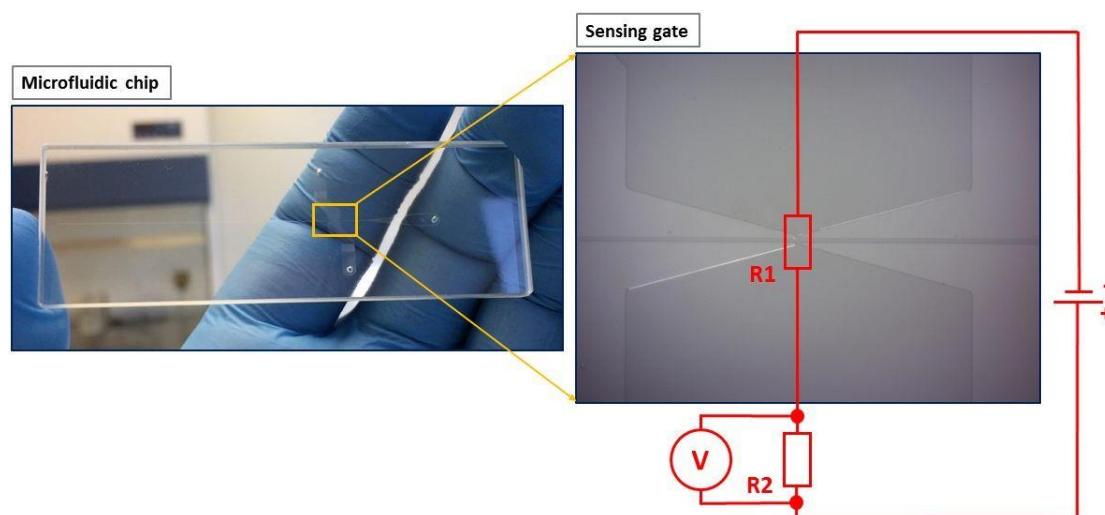


Fig. 1. Design of microfluidic chip and representation of sensing circuitry.

3 Results and Discussion

Two designs of the sensing gate were tested. In the first design as a sensing gate was used 36 x 15 μm microchannel but 2 mm long. Experiments with several buffers of various ionic strength proved volume of this section to be excessively large to register *Saccharomyces cerevisiae* cells of a diameter approx. 5 μm . Therefore in the second design the sensing gate had dimensions of 50 x 36 x 15 μm so the entering cell causes significant displacement of conductive electrolyte thus causing a noticeable drop in voltage measured across the sensing gate.

4 Conclusions

Two designs of a cell counting unit based on resistive pulse sensing were tested for further integration into a microfluidic device for cellular analysis. Resistive pulse sensing based counting unit shows good potential for controlling of cellular input in miniaturized systems.

Acknowledgement

Financial support from the Grant Agency of the Czech Republic (16-09283Y, P206/12/G014), and the institutional research plan (RVO:68081715) is acknowledged.

P74 PREDICTION OF GRADIENT RETENTION DATA FOR HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHIC SEPARATION OF NATIVE AND FLUORESCENTLY LABELED OLIGOSACCHARIDES

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Summary

In this study, we have investigated mixed-mode retention model and its extended version for predictions of retention times of native and fluorescently labelled oligosaccharides separated in hydrophilic interaction liquid chromatography (HILIC) in gradient elution mode. Native oligosaccharides (maltose to maltoheptaose) were derivatized using four reagents: 2-aminobenzoic acid, 2-aminobenzamide, 2-aminopyridine and 8-aminonaphthalene-1,3,6-trisulfonic acid. The separations were realized on the column packed with amide-type stationary phase in two different mobile phases consisted of acetonitrile and water with 0.1% acetic acid (v/v) and acetonitrile with 30 mM ammonium acetate.

1 Introduction

For description and prediction of retention behavior in HILIC, the mixed-mode isocratic retention model can be used, given by following equation:

$$\ln k = a + b \ln \varphi_{H_2O} + c \varphi_{H_2O} \quad (1)$$

where a is parameter related to interaction energy between solutes and stationary and mobile phase, b is coefficient related to the direct analyte-stationary phase interaction and c is coefficient related to the interaction energy between solutes and solvents [1]. This model can be extended with parameter d , describing contribution of glucose unit to the retention of native and derivatized maltooligosaccharides, and it is defined as:

$$\ln k = a_2 + b_2 \cdot \ln \varphi_{H_2O} + c_2 \cdot \varphi_{H_2O} + d \cdot n_{Dp} \quad (2)$$

where a_2 , b_2 and c_2 parameters have similar meaning like in Eq. (1) for glucose (monomeric unit) and parameter d is contribution of the oligomeric unit to the retention [2]. These models are describing retention in isocratic elution, where the separation is realized by mobile phase with constant composition. In gradient elution, the mobile phase composition is changing over the time of analysis. For description of retention in gradient elution, Eqs. (1) and (2) were used for calculation of immediate value of retention factor of solute band at each volume element dV and numerically integrated.

2 Experimental

For gradient separations of native and derivatized maltooligosaccharides the Eksigent microLC 200 liquid chromatograph was used. Mobile phase consisted of acetonitrile/water with addition of 0.1% (v/v) of acetic acid or 30 mM ammonium acetate. The separations were realized on TSKgel Amide-80 column (100 x 1 mm, 5 μ m, 80 Å). Column was thermostated at 35°C. The flow rate was set to 0.05 mL/min and the injected volume of the sample was 0.5 μ L. Detection was realized by mass spectrometry using AB SCIEX 4500 QTrap instrument with the electrospray ionization (ESI) in positive and negative ion mode. The spectra were recorded in Enhanced MS mode in the range m/z 300 – 1700 using following condition: source temperature 400 °C, curtain gas 30, gas $\frac{1}{2}$ = 30/0, entrance potential 10/-10V, ion spray voltage 4500/-4500 V.

3 Results and Discussion

Two retention models were used (mixed-mode model and oligomeric mixed-mode model) for predictions of retention times in gradient elution of native and derivatized maltooligosaccharides. Retention times of native and derivatized maltooligosaccharides were measured at 15 different gradient profiles (φ_0 (ACN) = 0.05, 0.15, 0.25 φ_{end} (ACN) = 0.5, 0.7, 0.8) and t_G = 5, 7.5, 10 min.

The computer-assisted method development gradient liquid chromatography usually utilizes application of isocratic retention models, which describes the retention

of the compounds in a wide concentration range of mobile phase. It was time-consuming to construct isocratic retention models, therefore to speed up the process and to verify the predictive ability of the models used, the experimental gradient retention times were compared with calculated values based on both, isocratic and gradient input data.

4 Conclusions

Experimentally obtained retention times of all analytes were compared with predicted times. In general the mixed-mode model provided better results compared to the model considering retention of all oligomers, however, only one scouting gradient run is enough for calculation of model parameters in this case. The highest average deviation obtained for mobile phase with 30 mM ammonium acetate calculated for mixed-mode retention model was 10.22 % and 14 % for extended model, respectively.

Acknowledgement

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P75 COMBINED EFFECT OF TEMPERATURE AND CDO NANOPARTICLES TREATMENT ON *PICEA ABIES*

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Summary

Three-years old seedlings of *Picea abies* were pre-treated under two different temperatures (20×35 °C) and subsequently exposed to elevated concentration of CdO nanoparticles (CdONPs). Two-week exposure to airborne CdONPs of ecologically relevant size and concentration significantly reduced photosynthetic light use efficiency in plants as well as contents of photosynthetic pigments and saccharides. In contrary,

amino acid content was increased under CdONPs. These changes were further modulated by previous growth temperature.

1 Introduction

It is suggested that terrestrial ecosystems will be exposed to a number of abiotic stressors including more frequent, prolonged heat waves and drought periods in the future [1]. Furthermore, in urban and industrial areas, the plants are also often exposed to elevated concentrations of atmospheric pollutants [2,3], including engineered nanoparticles (NPs) [4]. NPs, which are particles having a size smaller than 100 nm in at least one dimension, occur naturally in the biosphere as a result of volcanic eruptions and hydrothermal activity [5]. Intensive burning of fossil fuels as well as the development and application of nanotechnologies in recent decades are, however, directly related to the additional release of NPs into the environment [6]. Effects of NPs have been described in a variety of microbial and aquatic organisms [7], although the general consequences of higher plant exposure to NPs remain unclear. This is due to the fact that the interactions between NPs, particularly atmospheric NPs, and higher plant species have been studied only in a limited number of cases [8]. Our previous results revealed that airborne CdONPs may diffuse through a stomatal aperture of spring barley and affect the amount of primary and secondary metabolites [9].

The aim of the study presented was to investigate effect of airborne CdONPs on physiology and metabolism of coniferous Norway spruce (*Picea abies* L. Karst.). In particular, the effect of duration of exposure to CdONP treatment and an interaction with growth temperature were studied.

2 Experimental

2.1 Preparation of CdO nanoparticles

CdONPs were generated continuously *in situ* in a hot wall tube flow reactor, using an evaporation–oxidation–condensation technique in which a ceramic crucible containing a small amount of bulk cadmium was placed inside the ceramic work tube of a vertically orientated furnace (Carbolite TZF 15/50/610). The formed CdONPs were diluted with a stream of air (20 L min⁻¹) and used for a whole experiment in dose-concentration chambers. The concentration of CdONPs in air was stable at $2.03 \pm 0.45 \times 10^5$ particles cm⁻³. A particle size was in the range of 7–60 nm.

2.2 Experimental design

In total 80 of 3-years old *P. abies* seedlings were pre-treated under 20 °C and 35 °C using laboratory growth chambers FS-SI-4600 (PSI, Brno, Czech Republic). After two weeks, seedlings were transferred into CdONPs dose-concentration chamber and kept at 20 °C. A half of seedlings was placed in a chamber with no CdONPs, the second half of seedlings was exposed to the elevated concentration of CdONPs for two weeks. During the whole experiment the plants were exposed to the irradiance of 500 μmol m⁻² s⁻¹ (12 hours per day).

Physiological and biochemical measurements were made after 7 and 14 days of CdONP treatment.

2.3 Physiological and biochemical analyses

Photochemical efficiency of photosystem II was assessed by an *in vivo* chlorophyll fluorescence measurement using a PAM-2500 fluorometer (H.Walz, Effeltrich, Germany). A composition and an abundance of amino acids were analysed in positive and negative modes using UltiMate 3000 high performance liquid chromatograph coupled with an LTQ Orbitrap XL high resolution mass spectrometer (HRMS) (Thermo Fisher Scientific, Waltham, MA, USA). Saccharides and tocopherols were detected by gas chromatograph coupled with mass spectrometry (TSQ Quantum XLS triple Quadrupole; Thermo Fischer Scientific, USA). The composition of chlorophylls and carotenoids was determined spectrophotometrically using an UV/VIS SPECORD 250PLUS spectrophotometer (Analytik Jena, Jena, Germany). See Večeřová et al. [9] for details.

3 Results and Discussion

While short-term exposure to CdONPs (7 days) led only to the significant reduction in maximum quantum yield of photosystem II photochemistry, maximum quantum yield of photosystem II photochemistry, non-photochemical quenching and efficiency of photosystem II were significantly reduced after long-term treatment (14 days). In addition, this effect was more pronounced in seedlings pre-treated at 35 °C as compared to those pre-treated at 20 °C.

Biochemical analyses revealed that contents of photosynthetic pigments, amino acids, and saccharides were substantially affected by growth temperature. On the other hand, short-term exposure to CdONPs had no effect on a composition of photosynthetic pigments as well as contents of amino acids and saccharides. In contrary, tocopherols were not affected by temperature neither short-term CdONP treatment. After long-term exposure to CdONPs photosynthetic pigments were significantly decreased; however, there were no differences between seedlings previously exposed to 20 and 35 °C. Amino acid content increased mainly due to increases in valine, leucine, isoleucine, and asparagine (more than ten times) as compared to seedlings grown under CdONPs free air. In contrary to physiological analysis, CdONP treatment induced greater changes in amino acids content in seedlings pre-treated at 20 °C than in those pre-treated at 35 °C. Long-term treatment by CdONPs changed a saccharide composition particularly due to the significant reduction of sucrose and increase of glucose and fructose. While total saccharide content was reduced by CdONP treatment in seedlings pre-treated under 20 °C, differences in seedlings pre-treated at 35 °C were not observed. Long-term of CdONPs exposure led to a minor reduction of α -tocopherol content with no differences between samples of different pre-treatment.

Increase in amino acids and decrease in saccharide content is in accordance with our previous study with spring barley [9] although there the most abundant amino acids were tryptophane and phenylalanine. Valine, leucine and isoleucine are associated with defences against pathogens indicating more susceptibility of CdONP treated seedlings to a potential biotic stress.

4 Conclusions

Airborne CdONPs of ecologically relevant size and concentration have a potential to influence physiology and biochemical composition of coniferous *P. abies*. These changes further increase with the duration of exposure to CdONPs and are modulated by previous growth temperature.

Acknowledgement

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<http://dx.doi.org/10.1016/j.envpol.2016.05.013>.

P76 IDENTIFICATION OF PROTEIN-PROTEIN BINDING INTERFACES BY USING PEPTIDE MICROARRAY TECHNIQUE

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Summary

Scaffold proteins work as hubs that control cell signaling via organizing the molecules into multiprotein complexes. The binding events within the signaling complexes are elusive, mainly due to absence of detailed knowledge of the interaction interfaces. Here, we present an application of peptide microarray approach that can be used for a high resolution mapping of the protein binding epitopes. Our results open up a possibility to design specific inhibitors of particular protein-protein interactions that could help to elucidate the cell signaling processes.

1 Introduction

Scaffold proteins mediate assembly of multiprotein complexes that play a crucial role in cell signaling pathways, ensuring specificity and efficiency of the signal relay. Single scaffold protein is often able to interact with multiple partners from various signaling cascades and the combination of signaling molecules bound to the scaffold determines function of the particular complex. Thus, scaffold proteins serve as signaling hubs, shaping the cellular information flow¹. To gain mechanistic insight into formation of the signaling complexes (i.e. which proteins can bind simultaneously / exclusively), it is essential to map binding epitopes for the interaction partners on the scaffold at high resolution. This is complicated to achieve with commonly used biochemical methods (e.g. mutagenesis followed by protein pull-down / CoIP).

2 Experimental

Many scaffolding proteins contain intrinsically disordered regions that are responsible for mediating variety of interactions and the binding epitopes within the unstructured regions are often defined by linear peptide motifs^{2,3}. To make use of this fact, we have synthesized 13 amino acid (aa) peptides (with the sliding window of 3 aa, i.e. with 10 aa overlaps), covering complete sequence of a hub scaffold protein Axin, and the peptides were printed on a glass slide to make a "peptide microarray". The arrays were incubated with a series of recombinant Axin binding partners and the positive interactions were detected using fluorescently labeled antibodies.

3 Results and Discussion

Using this experimental approach, we have identified interaction interfaces for 4 Axin-binding partners at high resolution (± 5 aa). Our results show that Axin usually contains multiple binding epitopes for a single interaction partner. While some of the epitopes detected were unique, number of detected epitopes were shared among several interacting proteins from different signaling pathways. This finding indicates that the scaffold platform can't serve for all the pathways simultaneously and the competition between signaling proteins for the binding site on Axin could affect the pathway activities.

4 Conclusions

On the example of Axin, we have shown that the peptide microarray technique is a high-throughput method for identification of protein-protein interaction epitopes at high resolution. Precise knowledge of the binding interface allows a design of specific inhibitors of particular protein-protein interactions, which can be used for detailed investigation of cell signaling events.

Acknowledgement

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