Bi7430c MOLECULAR BIOTECHNOLOGY

practice

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01 course information

COURSE MOTIVATION

The practice provides an interesting combination of practical problems solved under the guidance of experts from academia (Masaryk University and Veterinary Research Institute) and business environment (a biotechnology company Enantis). At the end of the course, students will be familiar with a variety of techniques of molecular biotechnology, such as design of primers, DNA cleavage by restriction enzymes, transformation of bacterial cells, methods of fermentation culturing E. coli, application recombinant microorganisms and isolated enzymes in biosensing and remediation of hazardous substances or synthesis of pharmaceutically pure chemicals. The course will enable students to deepen their theoretical knowledge acquired in lectures Molecular Biotechnology. Students will become familiar with the currently used methods and the principle and operation of modern laboratory equipment. The course also aims to strengthen communication skills of students in a discussion with experts of the field and exercise stylistic skills of students in a writing an essays and protocols. The learning materials in English support the ability to work with scientific texts, including strengthening the knowledge of the English terminology used in this field.

CAPACITY two groups of 10 students

LANGUAGE materials – EN

spoken language – CZ

protocols – student chooses between EN and CZ

ABSENCE official excuse in the Information System (IS) + written essay (2 pages A4)

INTERACTIVE SYNOPSIS available in Bi7430c in the Information System

PREREQUISITES

1. parallel attendance of Molecular Biotechnology lecture (Bi7430)
2. the course of Molecular Biology (Bi4020) and Basics of Molecular Biology (Bi4010) completed

PRACTICE ORGANISATION

1. theoretical introduction (presentation given by lecturer)
2. introduction to practice and assignment control (homework)
3. experimental work in the laboratory

TEACHING AND ASSESSMENT METHODS

Students are expected to study the manual and prepare the homework before each practice. In the laboratory, each student works independently or in pairs under the guidance of the teacher. Each student works with own sample. The results of experiments are evaluated by each student in separate essays and protocols based on the individual practice. Students will obtain positive assessment of the practice for active participation in exercises and preparation of protocols or essays from each exercise. Protocols should be uploaded to IS within one week from each practice. Delay would result in extra written essay (1 page A4).

syllabus

PREPARATION AND TESTING OF MICROFLUIDIC CHIP

Lecturer: Mgr. Tomáš Buryška
Aims: fabrication of PDMS chip, preparation and microscopy of microdroplets, microscopy of microdroplets with cells

FERMENTATION OF RECOMBINANT MICROORGANISMS

Lecturer: Mgr. Lukáš Chrást
Aims: fermentation of *E. coli* biomass and expression of recombinant protein, technology EnBase

PREPARATION OF ENZYMATIC BIOSENSOR

Lecturer: Mgr. Šárka Bidmanová, Ph.D.
Aims: co-immobilization of recombinant enzyme and fluorescence probe to optical transducer, detection of selected analyte

BIODEGRADATION OF ENVIRONMENTAL POLLUTANT BY RECOMBINANT BACTERIUM

Lecturer: Mgr. Lukáš Chrást
Aims: engineered bacterium, synthetic metabolic pathway, biodegradation of environmental pollutant, analysis of metabolites

BIOCATALYTIC PREPARATION OF PHARMACEUTICAL PRECURSOR

Lecturer: Mgr. Veronika Štěpánková, Ph.D.
Aims: biocatalytic synthesis of pharmaceutical precursor by using recombinant enzyme

DESIGN OF RECOMBINANT SYSTEMS

Lecturer: Mgr. Andrea Schenkmayerová, Ph.D.
Aims: software tool Clone Manager, design of primers, restriction cleavage, cloning, transformation

PREPARATION AND TRANSFORMATION OF LIPOSOMES

Lecturer: RNDr. Jaroslav Turánek, CSc.
Aims: preparation of liposomes, transformation, high-pressure extrusion, preparation of SUV (small unilamellar vesicels), DLS (dynamic light scattering) analysis, ZetaSizer

ANALYSIS OF LIPOSOMES

Lecturer: RNDr. Jaroslav Turánek, CSc.
Aims: single particle tracking analysis by using Nanosight 500, transmission electron microscopy

workplaces

LOSCHMIDT LABORATORIES

http://loschmidt.chemi.muni.cz/peg/

The Josef Loschmidt Chair was established at the Faculty of Science of Masaryk University by a donation of Alfred and Isabel Bader to honour the name of great Czech scientist of the 19th century Jan Josef Loschmidt. Jiri Damborsky was appointed to the Chair in 2003 and the Loschmidt Laboratories were founded two years later. The Laboratories hold prestigious award from the European Molecular Biology Organization and the American foundation Howard Hughes Medical Institute. The research is focused to the areas of protein and metabolic engineering. Especially understanding the structure-function relationships of haloalkane dehalogenase enzymes and improve their functionalities for bioremediation, biocatalysis and biosensing.

ENANTIS, S.R.O.

http://www.enantis.com/

Enantis is a Czech R&D company founded in 2006 as the first biotechnology spin-off from Masaryk University, Brno, Czech Republic. Since 2008, the company has its own fully-equipped laboratories in the Biotechnology Incubator operated by the South Moravian Innovation Centre, where research, service and production operations are being carried out. Enantis provides consulting and development services in the field of enzyme technologies and protein engineering for biomedical, environmental, agrochemical and military-defence applications. Employing the latest technologies, the company offers its own range of products based on proteins and microorganisms.

VETERINARY RESEARCH INSTITUTE

http://www.vri.cz/

The Veterinary Research Institute in Brno (VRI) was founded in 1955. Collection of Animal Pathogenic Microorganisms which joined European Culture Collections Organisation in 1985 is a part of the Institute. The institution specializes in the veterinary medicine research. Its activity was focused on performing exact experiments with the aim to solve health problems in farm animals, protect people from zoonoses and to guarantee safety of foodstuffs and raw materials of animal origin.

02 lecturers

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Doc. RNDr. Zbyněk Prokop, Ph.D.

EDUCATION

He obtained his Master´s degree in Environmental Science and Ecotoxicology in 1998, Ph.D. degree in Environmental Chemistry in 2001 and habilitation from Environmental Chemistry in 2013 at the Masaryk University. Zbyněk Prokop extended his expertise in enzyme kinetics and microfluidics during his research stays at ETH Zurich in Switzerland, University of Cambridge in United Kingdom, University of Ghent in Belgium, University of Groningen in the Netherlands, EMBL and University of Hannover in Germany.

PROFESSIONAL ASSIGNMENTS

Zbyněk Prokop works as an associate professor in the Loschmidt Laboratories at Masaryk University were he leads a team engaged in enzyme mechanism and kinetics. He received the award for the Young Czech and Slovak Microbiologist of the Year 2002 of the Czechoslovak Society for Microbiology, the Excellence in Innovation Award of the Rector of Masaryk University in 2005, the Alfred Bader Prize for Bioorganic Chemistry of the Czech Chemical Society in 2005 and J. V. Kostir Prize for Biochemistry of the Czech Society of Biochemistry and Molecular Biology. He is co-author of 45 publications and three patents in the field of protein engineering and industrial enzymology. He is founder of a biotechnology spin-off company Enantis.

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Mgr. Šárka Bidmanová, Ph.D.

EDUCATION

Šárka Bidmanová received her master's degree in Microbiology and Secondary School Teacher Training in Biology in 2007 and Ph.D. degree in Environmental Chemistry in 2012 from the Masaryk University in Brno. She extended her expertise during her research stay at the Institute of Chemical Process Fundamentals at the Academy of Sciences of Czech Republic and at Institute of Analytical Chemistry/Chemo- and Biosensors at the University of Regensburg in Germany.

PROFESSIONAL ASSIGNMENTS

Šárka Bidmanová has been working as a researcher in Loschmidt Laboratories at Masaryk University since 2009. She is focused on the development, optimization and characterization of optical enzyme-based bioanalytical devices for environmental applications and military-defense. Her research interests also include immobilization and characterization of enzymes and pH indicators. She is co-author of eight publications and one book chapter. She obtained the Award of the Dean of the Faculty of Science of Masaryk University in 2013 and the Award of the Czechoslovak Microbiological Society in 2014.

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Mgr. Veronika Štěpánková, Ph.D

EDUCATION

Veronika Štěpánková received her master's degree in Biochemistry in 2008 and a Ph.D. degree in the same field in 2013 from the Masaryk University in Brno. She has obtained an international experience, theoretical knowledge and practical skills during a four months research visit at the University of Groningen in the Netherlands and several international courses.

PROFESSIONAL ASSIGNMENTS

Veronika Štěpánková has been working as a researcher in Loschmidt Laboratories at Masaryk University since 2008. Her research interests are particularly related to the biocatalysis using non-conventional media, such as organic solvents and ionic liquids, and biocatalytical production of enantiopure compounds. During her scientific carrier, she was author or co-author of eight publications, three book chapters and one patent. She was awarded the Jean-Marie Lehn Prize for talented young chemists (2nd place) in 2012. In 2013, she received the Award of the Dean of the Faculty of Science of Masaryk University and the Prize of the Czech Ministry of Education.

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Ing. Andrea Schenkmayerová, Ph.D.

EDUCATION

Andrea studied Biotechnology at the Slovak University of Technology in Bratislava, Slovakia and obtained her PhD in Biotechnology while doing research at the Slovak Academy of Sciences in Bratislava. Her studies were focused on fermentation and biotransformation processes, whole-cell immobilization techniques and construction of biosensors.

PROFESSIONAL ASSIGNMENTS

After finishing her PhD she was awarded a Claude Leon Fellowship for her two year PostDoc in Biochemistry at Stellenbosch University in South Africa. She was doing research on new antistaphylococcal drug targets. She is currently doing her second PostDoc in Protein Engineering at the Masaryk University in Brno, Czech Republic, being employed by the International Clinical Research Center of St. Anne's University Hospital.

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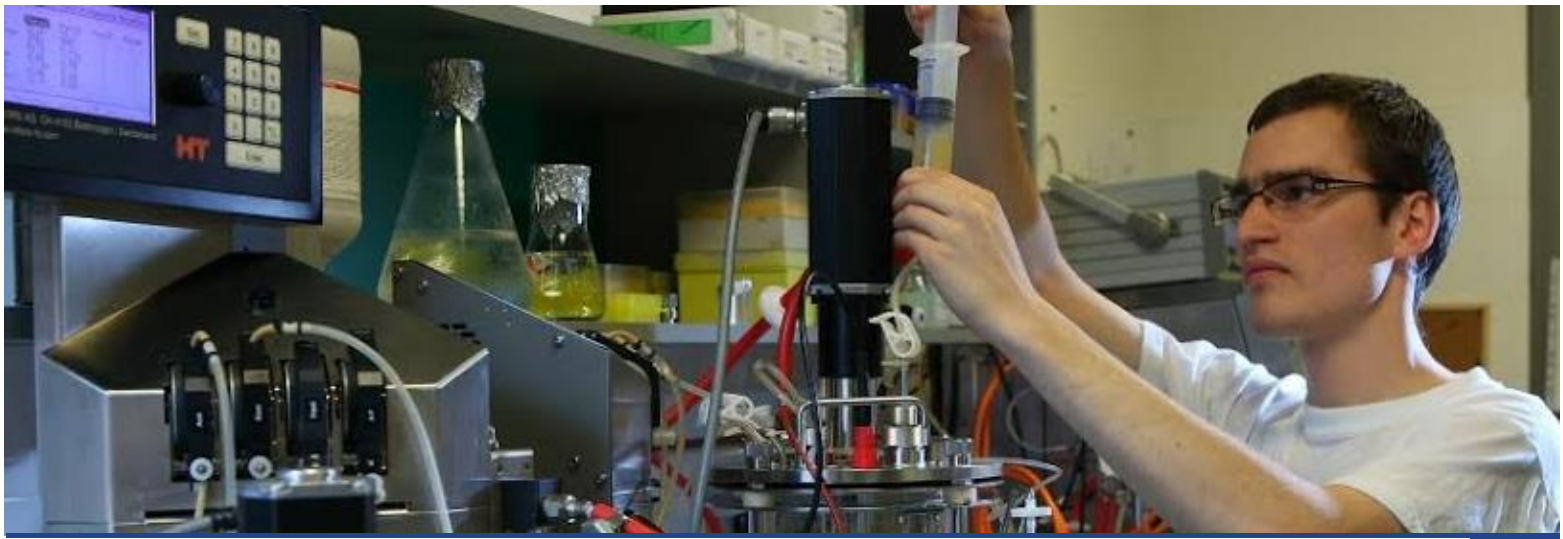
Mgr. Lukáš Chrást

EDUCATION

Lukáš Chrást received his master's degree in Microbiology in 2012. He is currently studying for Ph.D. degree in Microbiology at Masaryk University. He extended his experience with fermentation and cultivation techniques during internship in Contipro a.s., short stay at Institute of Microbiology of The Czech Academy of Sciences and during his three month stay at Institute for Biotechnology at Berlin Technical University in 2013. For the latter, he received Research Grant from the Federation of European Microbiological Societies.

PROFESSIONAL ASSIGNMENTS

Lukáš Chrást started in Loschmidt Laboratories as Master Student in 2010 and focused on characterization of haloalkane dehalogenases from extremophilic microorganisms. His current research interests are metabolic engineering of bacteria for biodegradation of halogenated compounds and fermentation technology. He is co-author of one research paper. He is member of Czechoslovak Society for Microbiology and Czech Biotechnology Society.



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Mgr. Tomáš Buryška

EDUCATION

Tomáš Buryška received his master's degree in Biochemistry in 2013. He is currently Ph.D. student of Microbiology at Masaryk University in Brno. He gained substantial part of his knowledge on a six month stay at the University of Cambridge in United Kingdom and during shorter stays at the University of Southampton and Korean Institute of Science and Technology in Germany.

PROFESSIONAL ASSIGNMENTS

Tomáš Buryška has been working in the Loschmidt laboratories at Masaryk University since late 2009. His specialization lies in the high-throughput methods, microfluidics, protein engineering and enzyme kinetics. He gained experience in projects focused on identification of novel enzyme substrates and inhibitors identified by *in sillico* methods. Since 2011 he intensively focuses on application of microfluidics in life sciences. Tomáš Buryška is a co-author of four research articles and one review article.

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RNDr. Jaroslav Turánek, CSc.

EDUCATION

He graduated from the Faculty of Science at Masaryk University, specialization biochemistry, and obtained the degree RNDr. in 1982. In 1987, he terminated post-graduated studies in the field of biochemistry and physical chemistry at the Faculty of Science, MU and South Bohemian Biological Centre, and obtained the degree CSc. Jaroslav Turánek extended his expertise during a research stay at GTC, Imperial College in London and by taking a number of specialist courses.

PROFESSIONAL ASSIGNMENTS

Jaroslav Turánek is Head of the Department of Pharmacology and Immunotherapy, Veterinary Research Institute, Brno, Czech Republic. His current research interests are development of recombinant vaccines and molecular adjuvants, immunotherapy of infectious diseases and cancer, nanodelivery systems for drugs (e.g., liposomes, micelles and dendrimers), and *in vivo* and *in vitro* pharmacological and toxicological models. Dr. Turánek is a lecturer of the following courses: Advanced Immunology (Masaryk University), Immunochemistry (Technical University), and Cellular Biotechnology (Technical University). He is a member of the Committee for state examination (Masaryk University) and supervisor of postgradual and pregradual students.

03 manuals

PREPARATION AND TESTING OF MICROFLUIDIC CHIP

location: INBIT, Kamenice 34, ground floor, room 023

lecturer: Mgr. Tomáš Buryška (bary@mail.muni.cz)

I. WORKFLOW

- preparation microfluidic chip
- basic chip operation
- droplet microfluidics and microscopy

II. MOTIVATION

Microfluidics can be defined as the science and technology manipulating and analysing fluid flow in sub-millimeter dimensions. It is becoming important technology for many emerging applications and disciplines, especially in the fields of chemistry, biology and medicine. Concrete application examples are biosensor devices for molecular diagnostics, polymerase chain reaction chips, high-throughput screening, controlled drug delivery systems, drug discovery methods, forensic analysis instruments, and so on (1).

III. THEORETICAL BACKGROUND

Formation of water in oil droplets in microfluidic chip has several benefits when compared to standard high-throughput technology. Amongst such benefits belong low volume of reagents consumed, chip modularity, low cost and simple fabrication. When all pros combined properly one may encounter drop costs of screening million fold (2, 3).

In this practice students will put hands on microfluidic chip fabrication on their own. During the lesson they will go through cutting out and assembling a chip applying oxygen plasma bonding. Prepared chips are to be used for water in oil droplet generation (4). An example will follow with encapsulation of single E.coli BL21 DE3 cells to droplets. Finally, there will be observation of single cells in emulsions generated.

IV. DESIGN OF EXPERIMENT

Solutions and reagents:

- Dow Corning Sylgard® 184
- HFE-7500 oil
- Trichloro(*1H,1H,2H,2H*-perfluorooctyl)silane
- 1.5M NaCl
- deionized distilled H2O

– *E.coli* BL21 DE3 or other bacterial cells
– Percoll ®
– isopropanol

Equipment:

– Chemyx Fusion 200 syringe pumps
– gas-tight syringes (various volumes)
– PTFE tubing
– razor
– biopsy puncher
– glass slide
– USB-microscope

Protocol for PDMS chip assembling:

1. using razor carefully cut PDMS with chip out of plate
2. with the help of ruler cut out single chip and place them bottom up
3. punch holes for inlet and outlet holes with biopsy puncher
4. clean glass slide and PDMS chip with isopropanol
5. place chip on the tray and put inside the oxygen plasma
6. run oxygen plasma for 3 minutes at 50% generator power at oxygen flow around 5scsm at 200 mTorr
7. immediately after oxygen plasma process ends, bond microfluidic chip and glass cover and press with your fingers; NOTE: try to avoid having bubbles between PDMS and glass
8. place bonded chip on hot plate at 60°C for another 5 minutes
9. let chip cool down to room temperature
10. flush channels with 1 % silane solution in HFE-7500, after 3 minutes replace by pure HFE-7500 oil and air

Droplet formation protocol:

1. load syringes with HFE-7500 oil and 150 mM NaCl, 25 % (v/v) Percoll and properly diluted cells
2. attach PTFE tubing to the syringe and remove any bubbles
3. put syringes into the syringe pumps, lock tight and set proper syringe diameter
4. connect syringe via tubing into the chip
5. set liquid flow – 300 µL.h-1 for oil phase and 30 µL.h-1 for the aqueous phase
6. observe droplet formation under microscope at various magnification
7. verify cell occupation in emulsions on inverted microsope

V. HOMEWORK

1. Estimate volume in pico-/femto- litres for monodisperse droplet formed at channel having dimensions 5, 10 and 20 µm, respectively (assume square cross-section forms spherical droplets of the same diameter).

2. For calculated droplet volumes estimate approximate cell density in $x.10^{y}$ per mL, to put single cell per droplet. There is approximately 1.10^{8} cells in medium with OD_{600} 0.5. Cultivated cells have OD_{600} 4.8. In case that grown culture has insufficient density, calculate the factor for thickening cell media to sufficient level. Account for 10 per cent pipetting error.

VI. LITERATURE

1. Dittrich, P. S., and Manz, A. (2006) Lab-on-a-chip: microfluidics in drug discovery., Nat. Rev. Drug Discov. 5, 210–8.
2. Mazutis, L., Gilbert, J., Ung, W. L., Weitz, D. a, Griffiths, A. D., and Heyman, J. a. (2013) Single-cell analysis and sorting using droplet-based microfluidics., Nat. Protoc. 8, 870–91.
3. Yin, H., and Marshall, D. (2012) Microfluidics for single cell analysis., Curr. Opin. Biotechnol. 23, 110–9.
4. Teh, S.-Y., Lin, R., Hung, L.-H., and Lee, A. P. (2008) Droplet microfluidics., Lab Chip, The Royal Society of Chemistry 8, 198–220.

FERMENTATION OF RECOMBINANT MICROORGANISMS

location: Loschmidt Laboratories, Kamenice 5/A13, 2nd floor, room 206
lecturer: Mgr. Lukáš Chrást (lukchrast@gmail.com)

I. WORKFLOW

- assembly of fermentation vessel
- calibration of probes
- setting up the fermentation
- sampling of cells from the fermentor
- downstream processes

II. MOTIVATION

Bioreactors (also known as fermentors or fermenters) are devices for cultivation of microorganisms playing important role in research and especially production of recombinant proteins or biomass in food and pharmaceutical industry. They can be operated under batch, fed-batch or continuous conditions, while fed-batch cultures are the most abundant in industrial applications. With high cell density cultures, great yields of recombinant proteins can be achieved, which can lower the expenses and therefore also the prices of final products.

III. THEORETICAL BACKGROUND

Bioreactor operation is very complex issue where several factors play crucial role on the performance of the culture. Composition of cultivation broth, sterilization efficiency, proper agitation and aeration, temperature or feeding strategy can all together influence final yield of biomass and recombinant protein from the fermentation. Since culture in fermentor is half opened system, it is very susceptible to contamination. It is very important for the operator of the fermentor to maintain sterility throughout the fermentation. In this practice, the students will learn how to assemble the fermentor vessel and prepare it for sterilization, how to calibrate pH and DO (dissolved oxygen) sensors and set up the fermentation together with creation of recipes for fed-batch cultivations. During the second half of the practice, they will aseptically take culture samples from the fermentor, evaluate cell density of the culture and perform the spread plate method for determination of the number of living cells in the culture. At the end, downstream processes such as membrane filtration and centrifugation will be applied to harvest cultured cells. The students will also learn how to determine wet and dry cell weight as important indicators of the state of the culture.

IV. DESIGN OF EXPERIMENT

Solution and reagents:

- *Escherichia coli* BL21(DE3)
- Luria-Bertani broth
- Plate Count Agar
- Struktol SB2020
- 10 % citric acid
- 10 % NaOH
- lactose
- PBS buffer
- 10% ajatin solution

Equipment:

- Biostat B Plus bench-top fermentor (Sartorius Stedim)
- Labfors 3 bench-top fermentor (Infors HT)
- table centrifuge
- ultracentrifuge Avanti J30I (Beckman-Coulter)
- LabStak M10 membrane filtration system (AlfaLaval)
- spectrophotometer and plastic cuvettes
- bench-top dryer STZ 5,4 (FALC)
- 10 mL syringes
- aluminum foil
- glass spreaders

Protocol for assembly of the fermentation vessel:

1. Prepare and dissolve all components of the fermentation broth. The volume of the medium should not be higher than working volume of the fermentor (usually 2/3 of total volume).
2. Calibrate pH probes (See Protocol for calibration of pH probes)
3. Pour dissolved medium to the vessel, add antifoaming reagent Struktol SB2020 to the final concentration 100 µL/L.
4. Insert baffles to the vessel. Make sure the O-ring is present on the top of the vessel.
5. Attach the lid to the glass part of the vessel by tightening large screws on the top of the lid.
6. Attach small plastic tubes to reagent inlet ports and sampling port, and big tubes to the sparger inlet and gas exhaust. Fix by tightening of screw clamps if necessary.
7. Attach the pH and DO probes to the vessel lid.
8. Place rubber septa to remaining ports and fix them with metal rings.

9. Close all plastic tubes with clamps, close sparger inlet tube with Hofman screw clamp.
10. Insert air filter to sparger inlet and gas exhaust tubing, cover ends with aluminium foil. Cover all plastic tube ends with alufoil.
11. Make sure all ports and tubes are closed, except for exhaust tube.
12. Sterilize the fermentor in autoclave.
13. After sterilization, let the fermentor cool down to room temperature.

Protocol for calibration of pH probe:

1. Connect the pH probe to the connector on fermentor control unit.
2. Select calibration mode on the control panel.
3. Put the probe in the container with calibration solution with pH 4.1.
4. Wait until the signal is stable, confirm the value.
5. Rinse the probe with distilled water.
6. Repeat measurement with calibration solution with pH 7 or 9.
7. Confirm new calibration slope.

Protocol for preparation of seed cultured:

1. Prepare night culture by picking one colony of freshly transformed *E. coli* BL21(DE3) cells to 10 mL LB medium with respective antibiotics.
2. Cultivate the cells overnight at 37 °C and 200 rpm.
3. Ca. 8-10 hrs prior to fermentation, prepare seed culture by transferring 2 mL of night culture to 200 mL of LB medium with respective antibiotics.
4. Incubate at 37 °C and 200 rpm.

Preparation of the vessel for fermentation protocol:

1. Fill up the jacket with tap water if necessary.
2. Connect the hose connectors to the thermostat.
3. Connect all probes to appropriate connectors on the control unit.
4. Turn on heating by setting the fermentation temperature (30 °C in our case).
5. Attach the motor on the top of the lid.
6. Wait until the DO probe is polarized.
7. Calibrate DO probe (See Protocol for calibration of DO probe).
8. Connect tubes to appropriate pumps and solution bottles (citric acid and NaOH).
9. Connect the sparger inlet tube to mass flow control.
10. Release all clamps on tubing with exception of clamp on sampling tube.
11. Set the cultivation parameters on control panel or in computer.
12. Inoculate fermentor with seed culture. For this purpose, seed culture volume corresponding to 1/100 of volume of medium in fermentor is used.

13. After 1 hr, add 2 g/L lactose to induce expression of heterologous protein.
14. Let the fermentation run overnight, harvest the cells by centrifugation and store the culture at -80 °C.

Protocol for calibration of DO probe:

1. Wait until the temperature of the medium reaches demanded temperature. Be sure the stirring is ON.
2. Connect bottle with pure nitrogen to sparger inlet tube via the sterile filter.
3. On control panel, select DO probe calibration module.
4. Wait until medium is fully saturated by nitrogen. Collect signal for 0 % O2.
5. Connect air tube to the sparger inlet.
6. Wait until the medium is saturated with air. Collect signal for 100 % O2.
7. Confirm new calibration slope.
8. This calibration has to be done prior to every fermentation! Oxygen solubility differs with medium composition and viscosity and its temperature.

Settings for batch fermentation:

1. Temperature 30 °C
2. Stirring cascade parameters: 30 % saturation, setpoint 500, minimum 500, maximum 1000 rpm
3. Aeration 0.5 vvm
4. pH 7

Protocol for sampling from the fermentor:

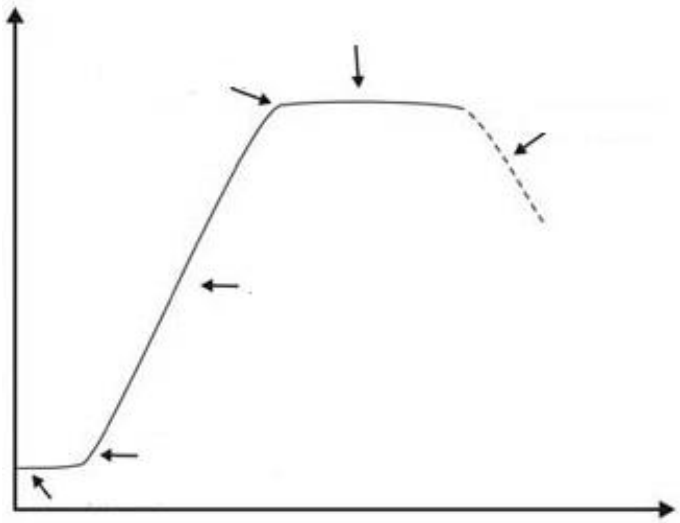
1. Connect the sterile syringe to sampling tube. Release the clamp. Suck ca. 5 mL to rinse the tube with fresh culture. Fix the clamp again. Unplug the syringe and discard whole volume. Repeate this to take your sample. Transfer the culture from syringe to sterile glass tube.
2. Transfer 1 mL of the culture to a plastic cuvette and measure optical density at 600 nm.
3. Prepare 6 tubes with 900 µL of PBS buffer. Transfer 100 µL of the culture to first tube, mix properly. Transfer 100 µL of suspension from first to second tube, repeat until the sixth tube. Take 100 µL of the final suspension and spread it with glass spreader on two plates with Plate Count Agar. Incubate the plates overnight at 37 °C.
4. Pre-weigh 15 mL plastic falcon, transfer 10 mL of the culture to the falcon and centrifuge for 5 min at 5,000 g. Discard supernatant and weigh the cells.
5. Resuspend the cells in 1 mL of water, transfer the suspension to pre-weighted aluminium foil. Put the foil with suspension for 2 hrs to dryer (110 °C). Weigh the foil again, calculate the dry cell weight.

V. HOMEWORK

1. Describe the difference between batch and fed-batch culture.

2. What does CFU/mL means? What method can be used to get these data?

3. Describe growth curve of bacterial culture. Label the phases and axes.



4. What are advantages and disadvantages of spectroscopic methods for determination of growth parameters of bacterial cultures?

VI. LITERATURE

1. Formenti, L. R., Nørregaard, A., Bolic, A., Hernandez, D. Q., Hagemann, T., Heins, A.-L., Larsson, H., Mears, L., Mauricio-Iglesias, M., Krühne, U., Gernaey, K. V. (2014) Challenges in industrial fermentation technology research. Biotechnol. J., 9: 727–738.
2. Junker, B. H. (2004) Scale-up methodologies for Escherichia coli and yeast fermentation processes. J. Biosci. Bioeng. 97: 347–364.
3. Kuprijanov, A., Schaepe, S., Aehle, M., Simutis, R., Lübbert, A. (2012) Improving cultivation processes for recombinant protein production. Bioprocess Biosyst. Eng. 35: 333–340.
4. Hoskinsson, P. A., Hobbs, G. (2005) Continuous culture – making a comeback? Microbiology. 151: 3153–3159.

PREPARATION OF ENZYMATIC BIOSENSOR

location: Enantis, s.r.o., INBIT, Kamenice 34 (4th floor, room 4.24)
lecturer: Mgr. Šárka Bidmanová, Ph.D. (bidmanova.s@seznam.cz)
Ing. Markéta Kotlánová

I. WORKFLOW

- preparation of biosensor discs
- preparation of reaction mixtures
- biosensor measurements
- evaluation of biosensor response
- calculation of detection limit

II. MOTIVATION

The need for simple, rapid, cost-effective, and portable screening methods has boosted the development of practical biosensors with applications in medical diagnostics, food safety, process control and environmental monitoring. Compared to traditional analytical methods, enzymatic bioanalytical devices have several distinct advantages such as high sensitivity and specificity, portability, cost-effectiveness, and the possibilities for miniaturization and mass production. Additionally, they can be developed for real-time and high-frequency testing without extensive sample preparation.

III. THEORETICAL BACKGROUND

Enzymatic biosensors employ the affinity and selectivity of catalytically active proteins, towards their target molecules. The transducer converts the effect created by the interaction of enzyme with the analyte, usually into an electrical signal. Depending on the assay type, two fundamental classes of enzymatic sensors can be distinguished. First, the enzyme detects the presence of a substrate, or co-substrate/co-factor. This is then, by way of a transducer, used to monitor the increase of enzymatic activity. The second group is based on the detection of inhibitors in the presence of a substrate. With this system the decrease of signal (caused by enzyme inhibition) is monitored. In this practice, optical biosensor based on enzyme haloalkane dehalogenase and fluorescent pH indicator will be introduced. Haloalkane dehalogenase catalyses conversion of halogenated hydrocarbons to a halide ion, an alcohol and a proton, the last being responsible for the signal change of fluorescence pH indicator. Described biosensor is useful for assessment of contamination at a particular environmental site or for monitoring the concentration of known halogenated contaminant.

IV. DESIGN OF EXPERIMENT

Solutions and reagents:

- enzyme haloalkane dehalogenase (EC 3.8.1.5)
- bovine serum albumin
- fluorescence pH indicator 5(6)-carboxynaphthofluorescein
- substrate 1,2-dibromoethane
- 1 mM HEPES buffer, pH 9.0: 1 mM HEPES, 20 mM Na_2SO_4, 1 mM EDTA
- 50 mM phosphate buffer, pH 9.0: 400 mM K_2HPO_4, 90 mM KH_2PO_4
- 25% (v/v) glycerol
- 70% (v/v) glutaraldehyde
- mixture of methanol with 1,2-dichloroethane as an internal standard

Safety precautions:

Glutaraldehyde, 1,2-dichloroethane and 1,2-dibromoethane are toxic chemicals. 1,2-dichloroethane and 1,2-dibromoethane are possibly and probably carcinogenic to humans, respectively. Therefore, these chemicals must be handled carefully under appropriate safety conditions. A fume hood with good ventilation is dedicated to preparation of the samples and biosensor measurements. The material is handled using appropriate personal safety equipment, including laboratory coats, glasses and gloves. All halogenated waste needs to be disposed separately.

Equipments:

- EnviroPen device with glass stick
- laboratory stand with a burette holder
- analytical balances
- pH meter with calibration solutions
- stirrer with stirring bar
- 10 ml vials, GC vials, vial crimper
- set of automatic pipettes
- glass discs
- Eppendorf tubes
- minishaker
- 10 microlitre syringe
- beaker
- graduated cylinder
- tweezer
- exsiccator
- computer with software FluorPen and Origin

Protocol for preparation of biosensor and measurement:

1. Weigh 4 mg of lyophilised enzyme and 8 mg of pH indicator in Eppendorf tube for preparation of enzymatic layer.
2. Weigh 4 mg of bovine serum albumin and 8 mg of pH indicator in Eppendorf tube for preparation of reference layer.
3. Dissolve each mixture in 65 µl of 25% glycerol by careful mixing.
4. Wash the glass discs with ethanol and distilled water.
5. Dry the glass discs with paper wipe.
6. Prepare enzymatic and reference discs - apply 5 µl of the corresponding mixture on each glass disc.
7. Expose the glass discs to vapour of 70% glutaraldehyde for 30 min.
8. Store the prepared glass discs in a Petri dish with 50 mM phosphate buffer, pH 9.0.
9. Put the glass disc with enzyme layer on the glass stick and attached into EnviroPen.
10. Switch on the device by pressing the SET key for 1 s.
11. Press SET key to measure Ft.
12. Press MENU key to scroll down into the Main menu.
13. Find Setting and adjust the F-fulse to 0.90 using SET key.
14. Press SET key to confirm the selection and MENU key to return to measurement.
15. Switch on the computer and run the program FluorPen.
16. Check that the program FluorPen and the EnviroPen device are properly paired.
17. Adjust the pH of 50 mM phosphate buffer to 9.0 using 1 mM NaOH if necessary.
18. Pipette 5 ml of this buffer into vial and add stirring bar.
19. Fix the EnviroPen device into the laboratory stand and immerse the tip into the vial with phosphate buffer.
20. Record the baseline while stirring for 10 min.
21. Dissolve chemicals for preparation of 1 mM HEPES buffer in 1 l of distilled water.
22. Adjust its pH to 9.0 using 1 M NaOH or 1 M HCl.
23. Pipette 5 ml of this buffer into vial and add stirring bar.
24. Inject 1 µl of substrate 1,2-dibromoethane into HEPES buffer and shake the reaction mixture on vortex (20 s).
25. Transfer the EnviroPen tip from phosphate buffer to HEPES buffer with substrate.
26. Record the enzymatic reaction with substrate for 2 min.
27. Save the measurement record using menu option File and Export in .txt format.
28. Remove the used glass disc.
29. Put a new glass disc with a reference layer on the glass stick.
30. Repeat the whole measurement procedure as described for disc with enzymatic layer.
31. Prepare sample for GC analysis – pipette 0.5 ml of methanol with 1,2-dichloroethane into GC vial.

32. Pipette 0.5 ml of 1 mM HEPES buffer with substrate to the methanol.
33. Cap the GC vial using vial crimper and shake the mixture on vortex (20 s).

Protocol for data analysis:

1. Copy the raw data into Excel file.
2. Plot the signal from enzymatic and reference layer in dependence on time of measurement in minutes.
3. Calculate a slope from the 1 min linear signal of enzymatic and reference layer.
4. Calculate biosensor response by substraction the reference slope from the enzymatic slope.
5. Plot the biosensor responses in dependence on substrate concentrations using software Origin and provided biosensor data.
6. Determine detection limit of biosensor for 1,2-dibromoethane.

V. HOMEWORK

1. Calculate amount of chemicals used for preparation of 1 l of 1 mM HEPES buffer: 1 mM HEPES (Mr 238.30 $g.mol^{-1}$), 20 mM Na_2SO_4 (Mr 142.04 $g.mol^{-1}$), 1 mM EDTA (Mr 292.24 $g.mol^{-1}$)

2. What is the concentration of 1,2-dibromoethane (Mr 187.86 $g.mol^{-1}$; ρ=2.18 $g.cm^{-3}$) if 1 µl of this chemical is dissolved in 5 ml of HEPES buffer?

3. Determination of halogenated pollutants using biosensor utilising haloalkane dehalogenase is based on measurement of:

A: enzyme inhibition

B: increase of product concentration

4. Which immobilization methods are commonly used in development of biosensors? What are their advantages and disadvantages?

5. What is the difference between selectivity and sensitivity of the biosensor?

VI. LITERATURE

1. Wencel D., Abel T., McDonagh C. (2014): Optical chemical pH sensors. Anal. Chem. 86: 15-29.
2. Long F., Zhu A., Shi H. (2013): Recent advances in optical biosensors for environmental monitoring and early warning. Sens. 13: 13928-13948.
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BIODEGRADATION OF ENVIRONMENTAL POLLUTANT BY RECOMBINANT BACTERIUM

location: Loschmidt Laboratories, Kamenice 5/A13, 2nd floor, rooms 208, 209, 227
lecturer: Mgr. Lukáš Chrást (lukchrast@gmail.com)

I. WORKFLOW

- whole-cell conversion of 1,2,3-trichloropropane to glycerol by recombinant *Pseudomonas putida* KT2440 with synthetic biodegradation pathway
- detection and quantification of produced glycerol using enzymatic kit
- analysis of samples from the reaction by gas chromatography (GC)

II. MOTIVATION

Bacteria and other microorganisms have great potential for biodegradation of polluting compounds (1). However, in case of some anthropogenic chemicals recently introduced into the environment, natural catabolic pathways in bacteria are not yet evolved toward their efficient degradation. Protein and metabolic engineering techniques can be applied in order to assembly synthetic metabolic pathways that might catabolize problematic substrates.

III. THEORETICAL BACKGROUND

In this practice, students will get the opportunity to work with recombinant bacterium *Pseudomonas putida* KT2440 carrying synthetic metabolic pathway for biodegradation of important environmental pollutant of anthropogenic origin 1,2,3-trichloropropane (TCP; 2,3). The pathway consists of three enzymes: haloalkane dehalogenase DhaA from *Rhodococcus rhodochrous* NCIMB 13064, and haloalcohol dehalogenase HheC and epoxide hydrolase EchA from *Agrobacterium radiobacter* AD1 (Figure 1). The activity of DhaA with TCP was improved 32-times by protein engineering resulting in mutant DhaA31 that is currently used in the pathway instead of wild-type enzyme (4). The whole pathway was assembled in form of synthetic operon and introduced on plasmid into the heterologous *host P. putida* KT2440. In the first part of the Practice, students will use cells of *P. putida* with expressed enzymes of the synthetic pathway to convert TCP to glycerol, a final product that accumulates in the cells during the initial phase of reaction. In the second part of the Practice, students will analyse samples from the reaction mixture and quantify TCP, glycerol and two other metabolites from TCP pathway, that accumulate during reaction, using enzymatic kit and GC.

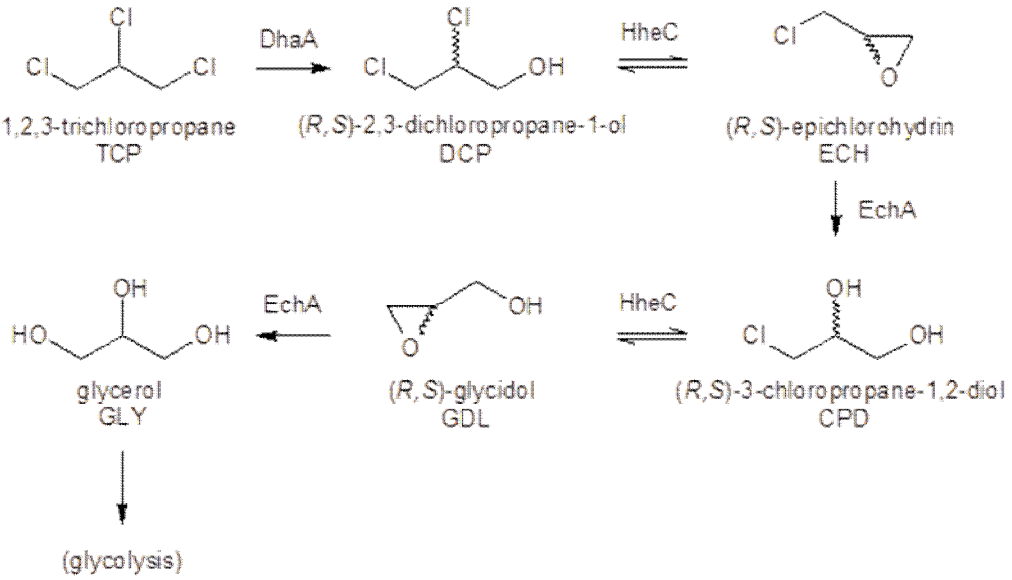


Figure 1. Scheme of the five-step synthetic metabolic pathway for biotransformation of 1,2,3-trichloropropane to glycerol. Used abbreviations of individual metabolites are shown. DhaA, haloalkane dehalogenase from *Rhodococcus rhodochrous* NCIMB 13064; HheC, haloalcohol dehalogenase; and EchA, epoxide hydrolase, both from *Agrobacterium radiobacter* AD1. The final product glycerol can be utilized in glycolysis when the pathway is assembled in vivo.

IV. DESIGN OF EXPERIMENT

Solutions and reagents:

- substrate 1,2,3-trichloropropane (Mr 147.43 g.mol-1, ρ=1.39 g.cm-3)
- sodium phosphate buffer (pH 7.0)
- quenching acetone with internal standard hexan-1-ol
- cell suspension of recombinant *P. putida* KT2440
- Free Glycerol Assay Kit (Biovision, USA) – buffer, enzyme mix, glycerol probe

Equipment:

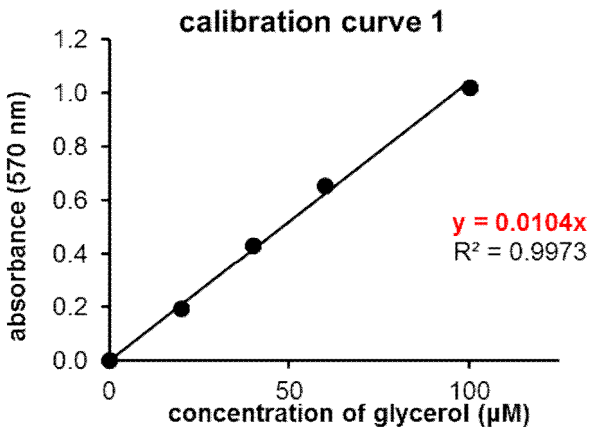
- gas-tight glass vials with with a screw cap mininert valve
- Hamilton syringe (1 ml) and pipettes
- glass vials (1 ml) with silicon septa caps for GC and cramping pliers
- Eppendorf tubes (1.5 ml)
- 96-well microtiter plates
- shaking water bath
- thermoblock
- microcentrifuge
- vortex
- UV-VIS spectrophotometer
- GC with flame ionisation detector and FFAP column

Protocol for whole-cell conversion of TCP (work in couples):

1. use 5 ml pipette and mix 5 ml of cell suspension with 5 ml of pre-incubated phosphate buffer with 2 mM TCP in gas-tight glass vials
2. start counting the time of reaction and incubate vials with shaking (150 RPM) in waterbath at 30°C
3. using Hamilton syringe, quickly withdraw 0.5 ml sample of reaction mixture and mix it with 0.5 ml of acetone in prepared Eppendorf tube, vortex for 10 s
4. withdraw 0.1 ml sample of the reaction mixture and boil it in Eppendorf tube for 10 min at 95°C in thermoblock
5. centrifuge both samples at 13000 RPM for 90s
6. transfer supernatant from sample with aceton to GC vial using pipette, close the vial with cap using cramping pliers
7. transfer the supernatant from the boiled sample to the new Eppendorf tube, keep the tube and GC vial for further analysis
8. repeat the procedure of sample collection every 10 min for 30 min, sign samples 0, 10, 20, and 30 min

Protocol for detection and quantification of produced glycerol:

1. use boiled 0.1 ml samples collected at times 0, 10, 20 and 30 min
2. pipette 50 µl of sample from 0 min into one well of the microtiter plate
3. pipette 25 µl of each of samples from 10 – 30 min at three separate wells and mix with 25 µl of kit buffer
4. add 50 µl of glycerol detecting pre-mix (2 µl of glycerol probe, 2 µl of enzyme mix, 46 µl of kit buffer) to each of four wells
5. incubate plate for 30 min in dark place at room temperature
6. analyse absorbance at 570 nm using UV-VIS spectrophotometer
7. use value from time 0 min as a blank and calculate concentration of glycerol in samples from 10, 20 and 30 min of the reaction, use slope from Calibration curve 1 for calculations (do not forget to include diluting factor)



Quantification of TCP and other metabolites from synthetic pathway by GC:

1. samples quenched in acetone will be analysed by GC with help of instructor
2. one sample will be analysed as a demonstration and raw data (peak areas of individual metabolites at certain time intervals) will be sent by instructor after the practice
3. calculate concentrations (mM) of TCP, 2,3-dichloropropan-1-ol (DCP) and glycidol (GDL) in reaction mixture using the slopes of calibration curves you prepared in advance (Homework 2) and raw data obtained from instructor, use internal standard to correct your data, do not forget to include diluting factor
4. write final concentrations of TCP, DCP and GDL in Table 1, check whether the sum of concentrations is always close to 2 mM

V. HOMEWORK

1. Calculate concentrations of metabolites from TCP pathway in reaction mixture.

Table 1. Concentrations of metabolites from TCP pathway in reaction mixture.

time	TCP	DCP	GDL	glycerol	SUM
0					
10					
20					
30					

2. Calculate how much of TCP (µl) has to be added to 5 ml of phosphate buffer to achieve final concentration of TCP in reaction mixture with recombinant cells equal to 2 mM. Remember, that 5 ml of phosphate buffer with TCP will be mixed with 5 ml of cell suspension to start the biodegradation reaction.

Result: **µl**

3. Use Microsoft Excel or alternative software. Prepare calibration curves (dependence of peak area on concentration of compound) for TCP and two metabolites that accumulate in the synthetic pathway during the biodegradation reaction. Use raw data from Table 2. Calculate a slope for each of three calibration curves.

Table 2. Peak areas of TCP, DCP and GDL determined for varying concentrations of compounds by GC.

conc. (mM)	TCP	DCP	GDL
1.250	86.0	87.0	37.0
0.625	45.0	44.0	19.5
0.313	22.0	22.0	11.0
0.156	11.0	11.0	5.4
0.078	5.6	5.6	2.8
0.039	3.0	2.8	1.3
0.020	1.6	1.5	0.7

Glue the final graph with three calibration curves and corresponding slopes here:

VI. LITERATURE

1. Copley, S. D. (2009) Evolution of efficient pathways for degradation of anthropogenic chemicals. Nat. Chem. Biol. 5, 559–66.
2. Bosma, T., Damborsky, J., Stucki, G., and Janssen, D. B. (2002) Biodegradation of 1,2,3-trichloropropane through directed evolution and heterologous expression of a haloalkane dehalogenase. Appl. Environ. Microbiol. 68, 3582–7.
3. Kurumbang, N. P., Dvorak, P., Bendl, J., Brezovsky, J., Prokop, Z., and Damborsky, J. (2014) Computer-assisted engineering of the synthetic pathway for biodegradation of a toxic persistent pollutant. ACS Synth. Biol. 3, 172–81.
4. Pavlova, M., Klvana, M., Prokop, Z., Chaloupkova, R., Otyepka, M., Wade, R. C., Tsuda, M., Nagata, Y., Damborsky, J. (2009) Redesigning dehalogenase access tunnels as a strategy for degrading an anthropogenic substrate. Nat. Chem. Biol. 5, 727–33.

BIOCATALYTIC PREPARATION OF PHARMACEUTICAL PRECURSOR (*S*)-2-BROMOPENTANE

location: Enantis, s.r.o., INBIT, Kamenice 34, 4th floor, room 4.24
lecturer: Mgr. Veronika Štěpánková, Ph.D. (veronika@chemi.muni.cz)

I. WORKFLOW

- preparation of reaction mixtures
- enantioselectivity measurements
- gas chromatography analysis
- calculation of enantiomeric excess and yield

II. THEORETICAL BACKGROUND AND MOTIVATION

Enzymatic enantioselectivity may be defined as the ability of an enzyme to distinguish between two enantiomeric substrates. The discriminating capacity of the enzyme is quantitatively measured by the ratio E of the corresponding specificity constants V_m/K_m (or k_{cat}/K_m). The enantioselectivity of enzymes has been exploited in organic synthesis, for example in kinetic resolution of racemic mixtures as well as in the synthesis of chiral building blocks from achiral precursors. Enzymatic kinetic resolution of racemic mixtures is an effective tool for the preparation of enantiomerically enriched compounds which is a continuous social demand due to the clinical advantages that enantiopure drugs offer over the racemic forms. Haloalkane dehalogenase DbjA from *Bradyrhizobium japonicum* USDA110 showed high enantioselectivity with β-substituted bromoalkanes. This enzyme is selectively acting on (*R*)-enantiomer, allowing the preparation of enantiopure (*S*)-enantiomer using kinetic resolution.

III. DESIGN OF EXPERIMENT

Solutions and reagents:

- glycine buffer (100 mM, pH 8.6)
- (*rac*)-2-bromopentane
- haloalkane dehalogenase DbjA (c = 1.5 mg/ml)
- diethyl ether

Equipments:

- 25-ml Reacti Flask with Mininert Valves
- automatic pipette 5 ml
- Hamilton syringe 1 ml
- test tubes
- glass vials with crimp caps
- Pasteur pipettes
- water shaking bath
- vortex mixer
- gas chromatograph equipped with a chiral column

Procedure:

1. Inject 10 µl of (*rac*)-2-bromopentane into 15 ml of glycine buffer in 25-ml Reacti Flask closed by Mininert Valves.
2. Prepare the required number of test tubes with aliquots of diethyl ether (1 ml).
3. Incubate the reaction mixture in a water shaking bath at the room temperature (approx. 20 °C) for 15 min.
4. Withdraw 1 ml from the reaction mixture and mix it with 1 ml of diethyl ether in the test tube to stop the reaction (sample at 0 min).
5. Vortex the sample for 30 s for the extraction.
6. Start the reaction by adding 0.5 ml of the enzyme solution (c = 1.5 mg/ml).
7. Withdraw 1 ml of the reaction mixture at 5, 10, 15 and 20 min and mixed samples with 1 ml of diethyl ether in test tubes. Do not forget to vortex each sample for 30 s for the extraction!
8. Transfer the ether (upper) part of the sample into a clean vial.
9. Analyse samples by gas chromatograph equipped with a chiral column.

IV. HOMEWORK

1. Calculate:
   a. concentration of enzyme (mM) in the reaction mixture
   b. theoretical concentration of substrate (mM) at the beginning of measurement
   c. enantiomeric excess (%) and yield (%) of (*S*)-2-bromopentane.

2. Prepare protocol.
3. Read article related to the topic of practice. The article can be found in study materials.

Protocol:

BIOCATALYTIC PREPARATION OF PHARMACEUTICAL PRECURSOR (*S*)-2-BROMOPENTANE

Name:

Date:

MOTIVATION:

EXPERIMENTAL PART:

RESULTS AND CALCULATIONS:

DISCUSSION:

V. LITERATURE

1. Faber, K. (2000). Biotransformation in Organic Chemistry, Springer-Verlag, Berlin.
2. Straathof, J. J., Jongejan, J. A. (1997). The enantiomeric ratio: origin, determination and prediction. Enzyme and Microbial Technology 21: 559-571.

IN SILICO CLONING OF A HALOALKANE DEHALOGENASE

location: Loschmidt Laboratories, Kamenice 5/A5 room 114 or A17/332
lecturer: Ing. Andrea Schenkmayerová, Ph.D. (andrea.schenkmayerova@fnusa.cz)

I. WORKFLOW

During this practical course/computer exercise we will perform all steps of the cloning of the haloalkane dehalogenase gene (*dhaA*) from *Rhodococcus rhodochrous*. The gene will be cloned in the pET21b vector for recombinant (over)expression in *Escherichia coli*.

- design of the primers for cloning the dhaA gene in pET21b
- *in silico* PCR reaction is performed using the gene sequence and the created primers to create the RE site containing DNA fragment
- the restriction digest is performed on both the vector and insert using the chosen restriction enzymes
- the two fragments are ligated to obtain the final plasmid
- finally the sequence obtained from sequencing is compared with the plasmid that was made in CloneManager [1]

II. MOTIVATION

An important step in biochemical research and biocatalytic processes is the production of the protein of interest. Therefore, the corresponding gene will be transferred into a vector (e.g. plasmid) which can be transformed into expression hosts. This exploitation of nature allows scientists to express their protein of interest at larger scale but also to alter it by mutagenesis or attach specific tags to facilitate purification.

III. THEORETICAL BACKGROUND

What is Molecular Biotechnology? In its broadest sense, molecular biotechnology is the use of laboratory techniques to study and modify nucleic acids and proteins for applications in areas such as human and animal health, agriculture, and the environment. Thus, molecular biotechnology is the branch of biology that deals with the formation, structure, and function of macromolecules essential to life, such as nucleic acids and proteins, and especially with their role in cell replication and the transmission of genetic information. Molecular biotechnology results from the convergence of many areas of research, such as molecular biology, microbiology, biochemistry, immunology, genetics, and cell biology (Figure 1).

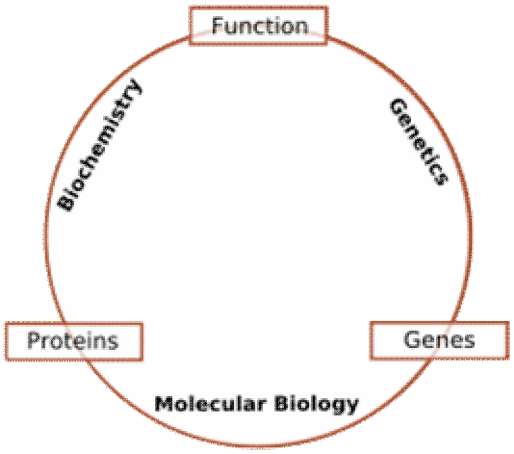


Figure 1. Schematic relationships the different research areas in Molecular biotechnology (biochemistry, genetics, and molecular biology).

More recently, much work has been done at the interface of molecular biology and computer science, namely in bioinformatics and computational biology. Molecular biotechnology is an exciting field fuelled by the ability to transfer genetic information between organisms with the goal of understanding important biological processes or creating a useful product. The central dogma (Figure 2) of molecular biology confirms the importance of genetic information or DNA (and RNA) in nature and the biological sciences.

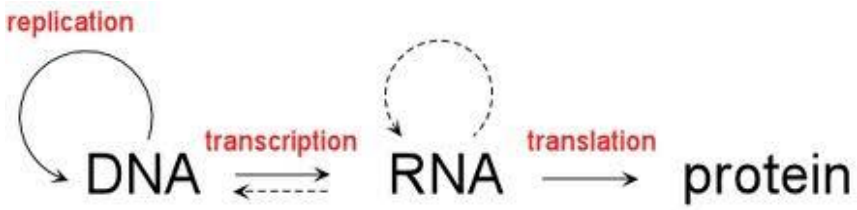


Figure 2. The Central dogma of molecular biology is an explanation of the flow of genetic information within a biological system

The transfer, use or changing of genetic information (manipulation of DNA) is a very important step – and in many cases the first experimental step – in biotechnological research since this enables to introduce, delete or alter the DNA and hereby change and study the characteristics of cells, proteins, enzymes, … Molecular biology is the branch of biology that deals with the manipulation of DNA so that it can be sequenced or mutated or used to study the biological effects of the mutation(s), gene product, …

In a standard molecular cloning experiment, the cloning of the desired DNA fragment can be seen as a seven step process (Figure 3). Several types of DNA manipulations are used here, including amplification, digestion and ligation by polymerase chain reaction (PCR), restriction enzymes and a ligase, respectively. Standard protocols for molecular cloning and DNA handling are well described in several (practical) handbooks [2].

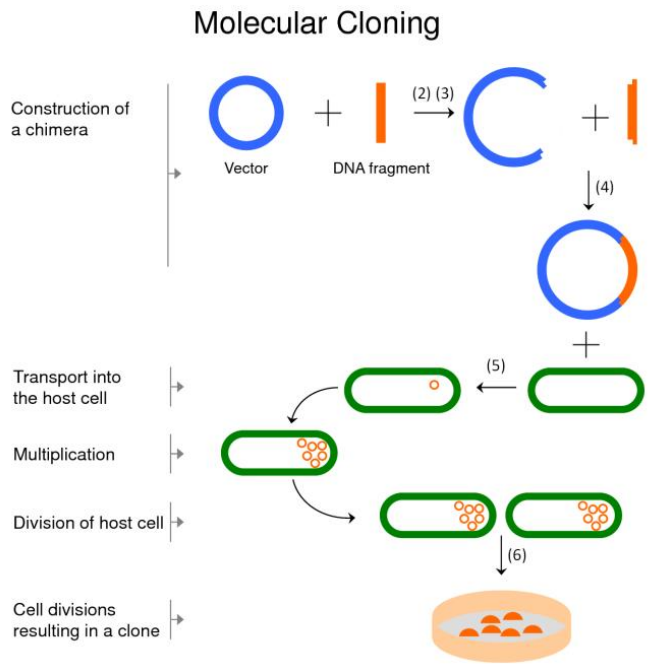


Figure 3. The different steps of a typical molecular cloning process. Numbers between brackets correspond to the seven step mentioned in the text.

The seven steps of a standard molecular cloning experiment

1. Choice of a host organism and a cloning vector

Several host organisms and molecular cloning vectors are available, however, for the vast majority of molecular cloning experiments a lab strain of the bacterium *E. coli* is used together with an appropriate plasmid cloning vector. *E. coli* and its vectors are widely used due to their relative simplicity, yet technically sophisticated nature, versatility, and wide availability. In addition, they offer rapid growth of recombinant organisms with minimal equipment.

2. Preparation of vector DNA

To prepare the cloning vector to accept the gene of interest it is treated with restriction endonucleases in order to create (specific) sites at which the foreign DNA can be inserted.

3. Preparation of DNA to be cloned

The gene of interest can be picked up from genomic DNA extracted from the organism of interest. Any tissues source can be used as long as the DNA is intact. In case of starting from RNA, a reverse transcriptase step is needed to create complementary DNA (cDNA). Nowadays, it is also possible to order synthetic DNA sequences, for example from hard to amplify fragments, for variants containing multiple mutations, eukaryotic genes (cfr. introns and RNA splicing) or unnatural sequences. Artificial gene synthesis has the advantage of to build in or removal of specific restriction sites as well as codon optimization for your specified host organism.

PCR methods (Figure 4) are the techniques used for amplification of specific DNA or RNA (RT-PCR) sequences prior to molecular cloning. While picking up the gene of interest one can simultaneously 'attach' the desired restriction sites to the PCR product by using primers containing these restriction sites.

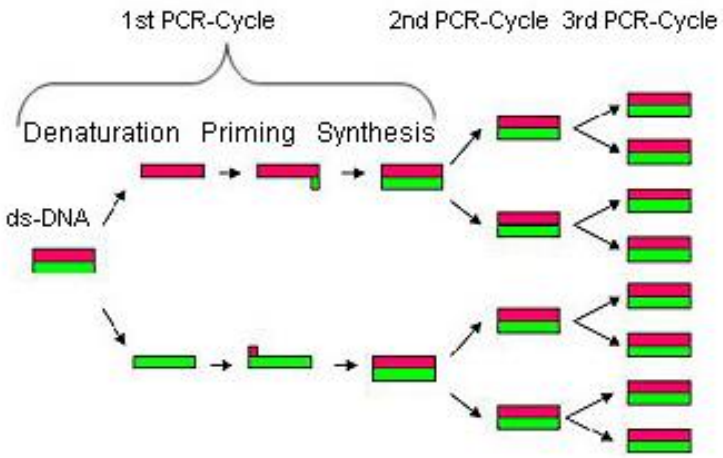


Figure 4. Amplification of a DNA fragment is achieved by PCR

4. Creation of recombinant DNA

In this step, the vector and foreign DNA are mixed at appropriate concentrations and an enzyme called DNA ligase will covalently link the ends together, creating a single DNA molecule which can be introduced into the host organism. Nowadays, new techniques have been developed like restriction/ligation free cloning, such as Gibson assembly [3] which allows for the joining of multiple DNA fragments in a single, isothermal reaction.

5. Introduction of recombinant DNA into a host organism

The *in vitro* prepared DNA can now be inserted back into a living cell, namely the host organism. Several options are available depending upon the experimental method and host cells (e.g. transformation, transduction, transfection, electroporation).

6. Selection of organisms containing recombinant DNA

The introduction of recombinant DNA into host organisms is usually a low efficiency process. Only a small number of cells will actually take up DNA. Therefore, efficient selection of the good clones is needed. When using bacterial cells, this can easily be achieved by a selectable marker on the vector. Most common are antibiotic resistance markers which allow the good clones to survive in the presence of antibiotic while the other cells die.

7. Screening for clones with desired DNA inserts and biological properties.

To make sure that the surviving colonies contain your gene of interest a very wide range of

experimental methods is available, including the use of nucleic acid hybridizations, antibody probes, polymerase chain reaction, restriction fragment analysis and/or DNA sequencing. DNA sequencing is also used to confirm the absence of unwanted mutations and correct integration of the DNA fragment.

PCR Primer Design Guidelines

(adapted from http://www.premierbiosoft.com/tech_notes/PCR_Primer_Design.html)

1. Primer Length: The generally accepted optimal primer length for PCR primers is 18-22 bp. This length is long enough to ensure adequate specificity and short enough for primers to bind easily to the template

2. Primer Melting Temperature (T_m): T_m is the temperature at which one half of the DNA duplex will dissociate to become single stranded and indicates the duplex stability. T_m in the range of 52-58°C generally produce the best results. The formula T_m = 2°C x (A+T) + 4°C x (G+C) is popular for its simplicity and roughly accurate prediction of oligonucleotide T_m.

3. Primer Annealing Temperature: Too high T_a will produce insufficient primer-template hybridization resulting in low PCR product yield. Too low T_a may possibly lead to non-specific products caused by a high number of base pair mismatches.

4. GC Content: The GC content (the number of G's and C's in the primer as a percentage of the total bases) of primer should be 40-60%.

5. GC Clamp: The presence of G or C bases within the last five bases from the 3' end of primers (GC clamp) helps promote specific binding at the 3' end due to the stronger bonding of G and C bases. More than 3 G's or C's should be avoided in the last 5 bases at the 3' end of the primer.

6. Primer Secondary Structures: Presence of the primer secondary structures produced by intermolecular or intramolecular interactions can lead to poor or no yield of the product. Interactions can be intramolecular resulting to hairpin formation or self-dimerization or intermolecular when different primers in the PCR mix form a dimer.

7. Repeats (repetitive presence of di-nucleotides) and Runs (long runs of a single base) should be avoided because they can misprime.

8. 3' End Stability: It is the maximum ΔG value of the five bases from the 3' end. An unstable 3' end (less negative ΔG) will result in less false priming.

9. Avoid Template Secondary Structure, Avoid Cross Homology, …

Luckily, these rules have been integrated in Molecular Biology software like CloneManager and free online primer design tools.

In the case of this cloning experiment, specific sites have to be introduced where the restriction enzymes (RE) can cleave the DNA for easy and correct fusing of the insert (gene of interest) and plasmid vector. Therefore, it is important to choose the right restriction enzymes for cloning and add the according RE sites to the primers. Some simple rules: the chosen restriction enzymes cannot cleave anywhere else in the insert or vector fragment, sticky ends are better than blunt ends, non-compatible ends are desired as they allow unidirectional cloning.

IV. DESIGN OF EXPERIMENT

Equipment:

– PC with CloneManager software
– sequence of DhaA and pET21b plasmid

Workflow:

1. design of the primers for cloning the *dhaA* gene in pET21b
2. *in sillico* PCR reaction is performed using the gene sequence and the created primers to create the RE site containing DNA fragment
3. the restriction digest is performed on both the vector and insert (created PCR fragment) using the chosen restriction enzymes
4. the two fragments are ligated to obtain the final plasmid
5. finally the sequence obtained from sequencing is compared with the plasmid that was made in CloneManager [1].

V. EXERCISES

1. Design primers for the cloning of the haloalkane dehalogenase gene (*dhaA*) from *Rhodococcus rhodochrous* in the pET21b vector for recombinant expression in *Escherichia coli*. Write down the primer and underline the restriction enzyme sites.

Fwd:

Rev:

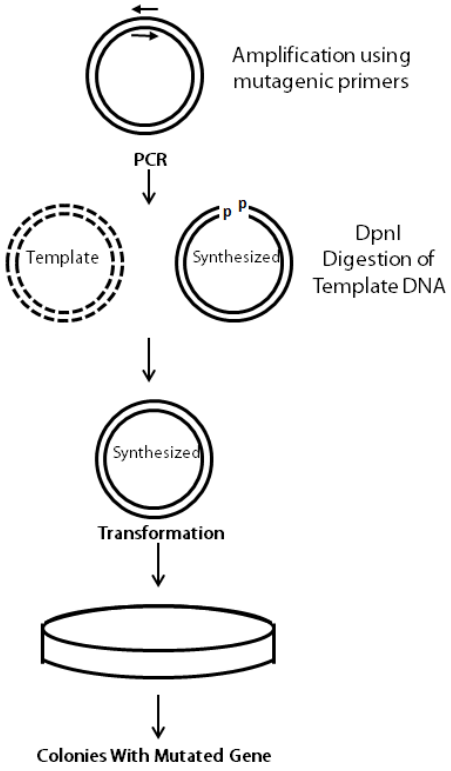
2. Calculate the melting temperatures of both primers using the A/T=2°C, G/C= 4°C rule and compare it with the melting temperatures found using CloneManager.

Check the New England Biolabs website (www.NEB.com) for more information about the restriction enzymes for question 3-5.

3. What is the advantage of using two different restriction enzymes for the cloning of a gene into a vector, for example *Nde*I and *Eag*I instead of *Nde*I at both ends? Which are the two problems that are avoided?

4. What is the advantage of using for example *Nde*I and *Eag*I for cloning rather than *Nae*I and *Bsr*BI?

5. Below you find a protocol for mutagenesis. You created a mutant library and accidently added *Dpn*II to the PCR mix instead of *Dpn*I. Do we have a problem now? Important to know: *E. coli* has a system for DNA methylation, which is not present in a PCR reaction.



6. You are provided with two sets of sequencing data (Fwd + Rev). Which one confirms the correct sequencing and which sample contains the error? What is the result of the error at DNA level?

VI. LITERATURE

1. CloneManager (SciEd software, USA), http://www.scied.com/pr_cmpro.htm
2. Green MR & Sambrook J (2012) Molecular cloning: A laboratory manual, 4th ed. (Cold Spring Harbor Laboratory Press, New York)
3. Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA, III, Smith HO (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nature Methods 6:343-345

PREPARATION AND TRANSFORMATION OF LIPOSOMES

location: Veterinary Research Institute, Hudcova 297/70, 2nd floor, Department of Pharmacology and Immunotherapy

lecturer: RNDr. Jaroslav Turánek, CSc. (turanek@vri.cz)

I. WORKFLOW

- preparation of liposomes
- transformation: high-pressure extrusion, preparation of SUV (small unilamellar vesicles)
- analysis: DLS (dynamic light scattering) analysis, ZetaSizer

II. MOTIVATION

Liposomes are very common delivery system in medicine because of their biocompatibility, non-toxicity, encapsulation of hydrophilic and hydrophobic compounds (e.g. drugs) and specific transport to the target region/area. Composition and surface of liposomes could be modified for better targeting, long circulation in the body or better stability. These modifications are a great tool for designing the liposomes according to current needs. After preparation a verification of the result is needed.

III. THEORETICAL BACKGROUND

Liposomes (closed bilayer phospholipid system) are known for more than 50 years and as delivery system they have many advantages. Their diameter is in the range of few nanometers to few micrometers. They can be unilamelar or multilamelar or even multivesicular and the bilayer is hydrophobic inside and hydrophilic outside. The process of assembling is not spontaneous like in micelles, it must be controlled and certain steps must be done. There are many possibilities of liposome preparation and many form to prepare, also there are some following steps to attached ligands to them or just make them more stabilized. In the method of Lipid film hydration the first step is to dissolve lipids in chloroform and this mixture evaporates by vacuum rotary evaporator. The resulting lipid film is hydrated by water PBS or other solution/buffer. Ultrasound or other techniques can help during hydration. At this point the liposomes are polydisperse and other process is needed to make them monodisperse.

Sonication and filtration through polycarbonate filters with defined pore size adjusts liposome diameter and lamilarity (extrusion). Also a method of freezing and unfreezing (FTMVL) can be used. Detergent removal method is used for preparation of unilamelar liposomes. When the detergent reaches its critical micelle concentration, it solubilizes lipids.

Dynamic light scattering is used for determining the size of small particles (proteins, polymers, micelles, carbohydrates, and nanoparticles) in suspension. It is also possible to measure the concentration and the size of surface. The principle is based on measuring the fluctuation of particles, when the smaller particle the bigger fluctuations.

TEM (transmission electron microscope) uses a beam for electrons transmitted through an ultra-thin specimen/sample. The electrons are interacting with the specimen and the resulting image is detected by a sensor/camera. TEM is able to detect and imagine at higher resolution than light microscope.

Preparation:

1. Lipid film hydratation
2. Detergent removal method
3. Sonication, extrusion, FTMVL

Analysis: DLS (size and polydispersity, zetapotential)

IV. DESIGN OF EXPERIMENT

Solutions and reagents:

- Lipids: 1-octadecanoyl-2-hexadecanoyl-sn-glycero-3-phosphocholine (SPPC), L-α-phosphatidylcholine (EPC)
- Sodium cholate
- NaCl
- Chloroform
- PBS
- Liquid N2

Equipments:

- vacuum rotary evaporator
- Liposofast
- Sonicator
- ZetaSizer

A) Lipid film hydration:

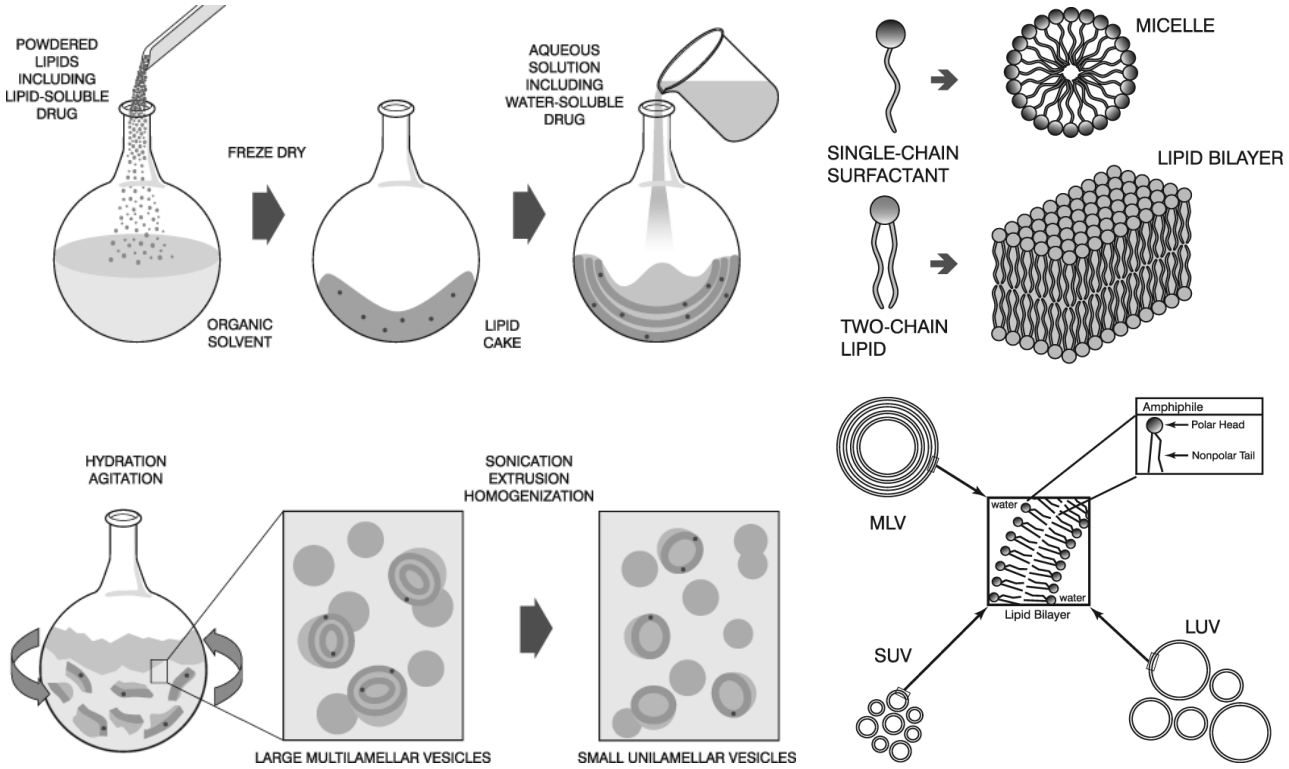
1. weight out: 100mg PPC-SPC
2. hydratation: 10ml 0,9% NaCl for infusio, final concentration of lipid: 10mg/ml

B) Detergent removal method

1. sodium cholate – dilution with amount up to critical micelle concentration (CMC)
2. dialysis

C) Sonication, extrusion, FTMVL

1. extrusion: filters 200nm (app. 15x)
2. sonication : total time 1 min, pulse mode: 10s pulse/10s pause, amplitude: 60%
3. FTMLV: frozen-thawed 2 times



D) DLS (Malvern) – size, size distribution, PDI

MLV, after extrusion, after sonication

V. LITERATURE

1. Avanti Lipids
   http://www.avantilipids.com/index.php?option=com_content&view=article&id=1384&Itemid=372

ANALYSIS OF LIPOSOMES

location: Veterinary Research Institute, Hudcova 297/70, 2nd floor, Department of Pharmacology and Immunotherapy

lecturer: RNDr. Jaroslav Turánek, CSc. (turanek@vri.cz)
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I. WORKFLOW

– single particle tracking analysis by using Nanosight 500– size, size distribution
– transmission electron microscopy (TEM) – size, morphology

II. MOTIVATION

Single particle tracking is a method for measuring the movement of every particle in a solution in time and for its quantifying. With this it is possible to study intracellular transport of these particles and their kinetics in real time. NanoSight instruments accurately and rapidly sort liposomes in water according to their size requiring only small volumes and very little sample preparation. The system enables individual liposomes in suspension to be visualized and their Brownian motion tracked. It anables specific and general nanoparticle tracking (presence, size distribution, concentration and fluorescence of all types of nanoparticles from 10nm to 2000nm depending on the instrument configuration and sample type). The particle size will determine the distribution of the particles within the body and the concentration and size of the particles will govern the amount of drug delivered.

III. THEORETICAL BACKGROUND

Single particle tracking is used to quantify specific behaviour of particles, e.g. liposomes, in colloid solution. The trajectory of every particle is measured and then the volume, hydrodynamic diameter and frequency of the particles are evaluated. This method is used to determine different kinds of particles in the mixture of variable materials.

The rate of movement is related only to the viscosity of the liquid, the temperature and size of the particle and is not influenced by particle density or refractive index. Absolute numbers of particles can be measured and the relative number of monomer versus aggregated particles is calculated in real time.

IV. DESIGN OF EXPERIMENT

Solutions and reagents:

– liposomes prepared in the previous exercise

Equipments:

– NanoSight 500

– TEM

A) NanoSight 500

Using the solution of lipids from previous exercise, dilution as needed

B) TEM

1. Preparation of specimen for TEM
2. Imaging

V. LITERATURE

1. NANOSIGHT: http://www.nanosight.com/technology/nanoparticle-tracking-analysis-nta

Protocol template

Practice XY

Name:

Date:

Motivation:

Experimental part:

Results:

Conclusions: