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Pharmaceutical Sciences

5th
PHARMA DOCDAY

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Plenary lecture

Prof. Dr. Dieter Steinhilber
Institute of Pharmaceutical Chemistry
Goethe University Frankfurt am Main

February 5th, 2015
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5th Pharma DocDay
February 5th, 2015

Time Schedule

9:30 – 9:45	Opening
9:45 – 10:30	**Plenary Lecture** **Prof. Dr. Dieter Steinhilber** – 5-lipoxygenase and leukemia development: Where are the links?
10:30 – 11:00	**Magdalena Taschwer** – Enantioseparation of cathinone and amphetamine derivatives by HPLC/UV using sulfated β-cyclodextrin as chiral selector **Anita Jerkovic** (ST) – Development of modified (bio)sensors for the determination of (oxidized) lipids
11:00 – 11:30	**Coffee break**
11:30 – 12:15	**Ramona Baumgartner** – NANEX: Process design with potential matrix materials **Karl Hörmann** (ST) – Drug delivery and drug targeting with intravenous lipid emulsions **Anna-Laurence Schachner-Nedherer** (ST) – Development of a nanoparticle-based miRNA delivery system for the treatment of obesity
12:15 – 13:15	**Lunch break**
13:15 – 14:15	**Michael Nagele** – Engineering of CXCL10 protein mutants as novel biotherapeutics **Andrea Feichtinger** (ST) – From ethnomedicine to drug optimization: Jacaranone-based leads to antiplasmodial und antitrypanosomal drugs **Mohammed Noureldin** – Cellular type-specific molecular mechanism of dihydrofolate reductase toward dihydrobiopterin
14:15 – 14:45	**Coffee break**
14:45 – 15:30	**Postersession:** **Jennifer Weiß** – Scientifically guided synthesis of N-methamphetamine versus internet tutorial **Miriam Russ** – Quantification of angiotensin-(1-7) in Karal®-solution with HPLC and fluorescence detection **Nikola Kitic** – Development of methods for the investigation of chemokine oligomerization **Diogo Gomes Lopes** – Structuring lipid coatings for modified drug release **Bernhard Scheicher** – Protamine titration process offers new possibilities in drug delivery via proticles
15:30 – 15:45	Closing remarks
15:45	Get together

ST = Shorttalk

Outline

Oral presentations

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Abstract 5th Pharma DocDay 2015

5-lipoxygenase and leukemia development: Where are the links?

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The ALOX5 gene encodes 5-lipoxygenase (5-LO), a key enzyme of inflammatory reactions. Regulation of 5-LO mRNA expression involves promoter activation and elongation control within the 3'-portion of the ALOX5 gene. The ALOX 5 gene is transcriptionally activated by trichostatin A (TSA). Physiologically, 5-LO expression is induced by calcitriol and/or TGFß which mainly involves induction of mRNA transcript elongation. We found that the H3K4 trimethylase MLL activates the ALOX5 promoter in a HDAC class I-dependent manner whereas the oncogenic MLL translocation product MLL-AF4 leads to a strong, HDAC-independent, constitutive 5-LO promoter activation. Interestingly, treatment of cells with HDAC class I inhibitors switch "inactive MLL" into "active MLL" and overwrites the dominant 5-LO promoter activation deriving from MLL-AF4. Furthermore, we found that the elongation factor AF4 supports VDR/RXR-dependent elongation of 5-LO mRNA transcripts by calcitriol and TGFß. Our data show that MLL and AF4 are involved in the regulation of ALOX5 transcript initiation and elongation, respectively.
Recently, 5-LO was shown to be critical to maintain cancer stem cell-like cells in a model of chronic myeloid leukemia. We investigated whether 5-LO is also involved in cancer stem cell maintenance in a model of acute myeloid leukemia. We found that pharmacologic inhibition of 5-LO interfered strongly with the aberrant stem cell capacity of PML/RARα-expressing hematopoietic stem cells. Our data suggest that 5-LO inhibition may be an interesting option for the treatment of acute myeloid leukemia.

Abstract 5th Pharma DocDay 2015

Enantioseparation of cathinone and amphetamine derivatives by HPLC/UV using sulfated β-cyclodextrin as chiral selector

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Over the past decade, a growing alternation of recreational drugs from natural materials and natural products to more potent and sometimes more harmful synthetic derivatives took place. Besides amphetamines, phenetylamines, benzofuries and tryptamines, cathinones developed to the most common abused compound class in the past 10 years.
These psychoactive compounds are mainly sold via online suppliers, which makes the handling of the worldwide black market difficult.
Since the majority of the analytes were not available from official suppliers, they were obtained from various online vendors. Before starting research, their identity was checked by GC-MS and if necessary by NMR.
Many of the novel psychoactive compounds possess a chiral center and are mainly traded as racemic mixtures. It is presumed that the enantiomers have different pharmaceutical potency as it is known from many pharmaceutical ingredients as well as of amphetamine. Thus, there is a demand to develop enantioseparation methods for these new recreational drugs.
This study presents the use of sulfated β-cyclodextrin in terms of HPLC as chiral additive to the mobile phase. Furthermore a comparison between a RP-18e and a RP-8e column by means of enantioseparation power was carried out. With this new method, chiral separation of a large spectrum of amphetamines and cathinones was performed [1]. Moreover, with the presented method elucidation of real life samples was feasible.

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Abstract 5th Pharma DocDay 2015

Development of modified (bio)sensors for the determination of (oxidized) lipids

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Lipid peroxidation is caused by oxidative stress and can damage lipoproteins, cell membranes and other lipid-containing structures, leading to ailments such as cardiovascular disease, cancer and neurodegenerative diseases like Parkinson´s and Alzheimer´s. [1, 2]
Polyunsatured fatty acids (PUFAs), such as arachidonic or linoleic acid, are major targets for lipid peroxidation and can be induced via non-enzymatic peroxidation by reactive oxygen species (ROS) or enzymatic peroxidation by the actions of lipoxygenases (LOXs) or cyclooxygenases (COXs). [3]
Lipoxygenases are capable of converting PUFAs into lipid hydroperoxides, which can be detected by biosensors. They are also capable of producing various signaling molecules, such as leukotrienes and lipoxins, known for their importance in inflammatory processes. [2]
Electrochemical biosensors have gained a lot of interest in the last decade, since they are simple and low-cost possibilities for the determination of bioactive substrates. [4]
In this work modified carbon paste (bio)sensors for the determination of lipids (PUFAs) and oxidized lipids (lipid hydroperoxides) were used. Carbon paste electrodes have the advantage that they show low background current, are easily modified and give fast responses. [4] A variety of modifiers and nanomaterials, e.g. nanotubes or gold-nanoparticles, as well as enzymes (5- LOX, 12-LOX or 15-LOX) will be investigated in order to improve and optimize the sensor system.

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Abstract 5th Pharma DocDay 2015

NANEX: Process design with potential matrix materials

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Recently, we presented the use of the NANEX process for developing a solid nano-formulation with an increased solubility behavior of the poorly water soluble model drug phenytoin incorporated in Soluplus® [1]. In the current study we focus on evaluating further matrix materials that are suitable for incorporation of aqueous suspensions during hot melt extrusion.
Kollidon® VA64 (KVA64), Eudragit® E PO (EEPO), AQOAT® (hypromellose acetate succinate, HPMCAS) and poly(ethylene glycol) 20000 (PEG 20000) were used for the NANEX experiments. All extrusion experiments were carried out using a MICRO 27 GL co-rotating twin-screw extruder (Leistritz Extrusionstechnik GmbH, Nürnberg, Germany). To assess whether the polymer could be mixed with water during the NANEX process and the added water could be removed completely via devolatilization (vacuum of 200 mbar), liquid (i.e., water) was fed to the hot melt extruder using the same experimental set-up as described previously [1]. The liquid was added to the extruder via a micro-angular gear pump in barrel 4, while feeding the matrix material in barrel 1 and degassing of the added water in barrel 8. By adding water to the extruder at fixed throughputs and screw speeds, their influence on the maximum amount of feedable water was examined. Clogging of the degassing unit and/or inclusions of moisture within the strands indicated the end-point. Additionally, the mean residence time at fixed process parameters was evaluated by adding a blue tracer via barrel 1. In this study two different screw configurations were employed according to the requirements of the used matrix material. Additionally, residual moisture contents of the extrudates were measured via Karl Fischer titration.
The results demonstrated that the amount of water fed to the material was strongly dependent on the filling degree of the screw, which is a function of the throughput and the screw speed. The lower the filling degree was the higher was the amount of added water. Similarly, the longer the residence time was, the more water could be added. However, the quantity of added and removed water was not only related to the process parameters but also to the nature of the matrix material. It was found that polymers, which are miscible with water could be processed via NANEX. Therefore, for HPMCAS/plasticizer, PEG 20000 and KVA64, water could be added to the molten materials to a variable extent.

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Abstract 5th Pharma DocDay 2015

Drug delivery and drug targeting with intravenous lipid emulsions

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Intravenous fat, also named oil or lipid, emulsions are in medical use since over 5 decades (e.g. Intralipid for parenteral nutrition, approved in Europe in 1962). This presentation of the review should show the latest research achievements to use this pharmaceutical formulation system:
as drug delivery system
for drug targeting.
By incorporation of lipophilic drugs into the oil droplets of an intravenously administered emulsion the biodistribution and pharmacokinetics can be changed to a beneficial way for the patients. Advantages which were achieved by the researchers are reaching from reduction of toxicity to increased bioavailability. Even with emulsion systems similar to parenteral nutrition emulsions a change in biodistribution to specific parts of the body (drug targeting) can be achieved.
Modification of the oil droplet surface can enhance the drug targeting effect manifold. PEGlyation for example increases the blood circulation time. Attaching ligands to this PEG chains like peptides can increase the selectivity or can aid for imaging technologies like NIRF due to adding a fluorescent dye molecule. An example is presented, where all of these features are combined for a “theranostic” emulsion1.

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Abstract 5th Pharma DocDay 2015

Development of a nanoparticle-based miRNA delivery system for the treatment of obesity

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Obesity is a major risk factor in the pathogenesis of the metabolic syndrome including type 2 diabetes, dyslipidemia and cardiovascular disease. These public health concerns present a therapeutic challenge and require novel therapeutic targets [1]. Therefore, the development of an effective drug delivery system targeting adipose tissue to treat obesity is of great interest.
MicroRNAs (miRNAs) are a class of non-coding endogenous small RNAs of approximately 22 nucleotides. They are involved in many different metabolic processes including lipid metabolism and adipocyte differentiation. MiRNAs regulate gene expression at posttranscriptional level by specific binding to their mRNA target. The researches of this thesis focus on miR-27a. This type of miRNA acts as a negative regulator of adipogenesis resulting in a suppression of adipogenic marker genes such as PPARγ [2-4]. Protamine and miR-27a form nanoparticles in a spontaneous self-assembled process based on electrostatic interactions between the positively charged peptide and the negatively charged miRNA. The carrier system should be able to protect miRNAs against enzymatic degradation caused by nucleases and promote cellular uptake and distribution. Physical properties of the miRNA-loaded nanoparticles like particle size, surface charge and miRNA protection against nuclease digestion are investigated. The miRNA delivery system is tested in vitro in comparison to commercial available transfection reagents such as Hiperfect using the two mice cell lines 3T3-L1 and 3T3-F442A. The expression pattern of miR-27a during adipogenesis and its effect on target mRNAs and protein levels in mature adipocytes are investigated.

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Abstract 5th Pharma Doc day 2015

Engineering of CXCL10 protein mutants as novel biotherapeutics

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Several acute and chronic inflammatory lung diseases like Chronic Obstructive Pulmonary Disease (COPD) or Idiopathic Pulmonary Disease (IPF) are characterised by infiltration of chemokine-activated leukocytes into the lung. [1]
CXCL10, a chemoattractant protein secreted in response to Interferon Gamma (IFN-γ) e.g. by endothelial cells or fibroblasts, seems to be one of the key players in the pathogenesis of these diseases. This small protein exerts its effects via the interaction with its corresponding receptor CXCR3, which is located on the surface of the targeted blood cells and via the interaction with glycosaminoglycans (GAGs) expressed on the surface of endothelial cells. [2]
The main focus of the underlying study lies on the development of biotherapeutics which interfere in this system and ultimately block the excessive inflammatory response of the human body. These biotherapeutics are so-called dominant, negative CXCL10 mutants: on the one hand, these mutants have a higher affinity towards the glycosaminoglycans and replace or at least compete with wild type CXCL10 and on the other hand, they have a knocked out GPCR activation ability and do not interact (or interact much less) with the targeted leukocytes. [1,2]
The first steps of the underlying study were in silico modelling of the mutants and establishing a suitable upstream- and downstream-processing procedure. Then we generated a fluorescently engineered CXCL10 variant which was subjected to biocomparability studies with the native, non-fluorescent protein.
Compared to the wild type, the fluorescently engineered mutant exhibited similar biological properties. Then we started to produce the first members of our nascent mutant library and tested their biophysically behaviour as well as their knocked out chemotactic potency on cells. These experiments included gel electrophoresis and Western Blot analysis to determine identity and purity; Circular Dichroism (CD) and chaotrop-induced unfolding to approximate structure, Isothermal Fluorescence Titration (IFT), Surface Plasmon Resonance (SPR) and Isothermal Titration Calorimetry (ITC) to quantify GAG-binding affinity and Boyden Chamber experiments to determine the chemotactic activity. The purpose, the experimental performance and the results of our recent work will be presented and described in depth in the underlying presentation.

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Abstract 5th Pharma DocDay 2015

From Ethnomedicine to Drug Optimization: Jacaranone-based Leads to Antiplasmodial und Antitrypanosomal Drugs

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Malaria and African trypanosomiasis remain one of the most devastating tropical diseases and affect millions of people around the world [1]. A major problem in combating these protozoal diseases concerns the increasing formation of drug-resistant strains. The search for novel lead compounds against these scourges of humanity is therefore of great importance [1,2].

Plant secondary metabolites provide a rich source for such lead compounds and drug development. During a screening of numerous plants traditionally used against protozoal diseases in South America, jacaranone and its glucosides were identified to possess strong and promising *in vitro* activity against *Plasmodium falciparum* and *Trypanosoma brucei rhodesiense* [3]

The final goal of the present work is the synthetic optimization of the jacaglabroside scaffold to enhance the antiprotozoal effect and further decrease its cytotoxicity (see graphics below).

Preliminary results suggested a beneficial impact of inserted halides in the aglycon part. In addition, the supplemental esterification of the sugar unit seems to be beneficial for antiprotozoal effect [4].

In order to evaluate the antiplasmodial, antitrypanosomal and cytotoxic activity, all synthesized compounds will be submitted to the *Swiss Tropical and Public Health Institute* in Basel.

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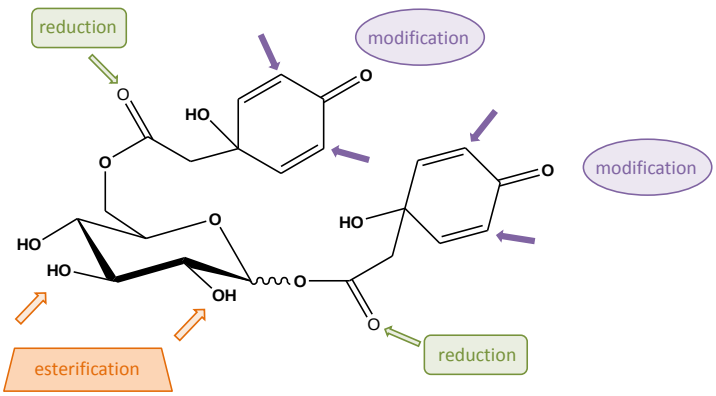
Graphics



Abstract 5th Pharma DocDay 2015

Cellular type-specific molecular mechanism of dihydrofolate reductase toward dihydrobiopterin

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(6R)-5,6,7,8-Tetrahydro-L-biopterin (BH4) availability regulates nitric oxide and superoxide formation by endothelial nitric oxide synthase (eNOS). At low BH4 or low BH4 to 7,8-dihydrobiopterin (BH2) ratios the enzyme becomes uncoupled and generates superoxide at the expense of NO. We studied the effects of exogenously added BH2 on intracellular BH4/BH2 ratios and eNOS activity in different types of endothelial cells. Incubation of porcine aortic endothelial cells with BH2 increased BH4/BH2 ratios from 8.4 (controls) and 0.5 (BH4-depleted cells) up to ~20, demonstrating efficient reduction of BH2. Uncoupled eNOS activity observed in BH4-depleted cells was prevented by preincubation with BH2. Recycling of BH4 was much less efficient in human endothelial cells isolated from umbilical veins or derived from dermal microvessels (HMEC-1 cells), which exhibited eNOS uncoupling and low BH4/BH2 ratios under basal conditions and responded to exogenous BH2 with only moderate increases in BH4/BH2 ratios. The kinetics of dihydrofolate reductase-catalyzed BH4 recycling in endothelial cytosols showed that the apparent BH2 affinity of the enzyme was 50- to 300-fold higher in porcine than in human cell preparations. Thus, the differential regulation of eNOS uncoupling in different types of endothelial cells may be explained by striking differences in the apparent BH2 affinity of dihydrofolate reductase.
Our current studies are aiming on identifying the molecular mechanism underlying the cellular types specific recycling of BH4.

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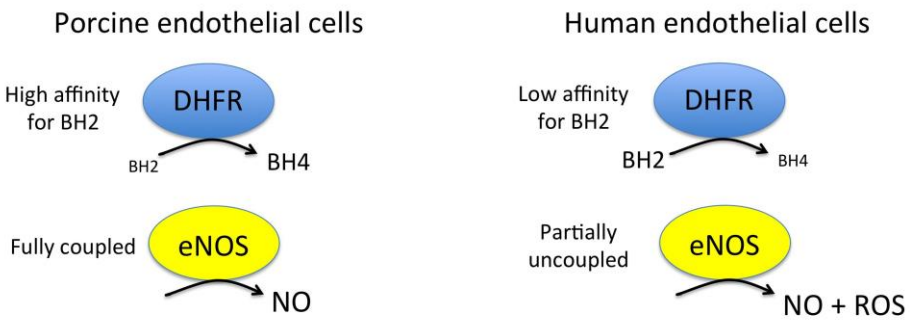
Graphics:



Abstract 5th Pharma DocDay 2015

Scientifically guided synthesis of N-methamphetamine versus internet tutorial

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The synthesis of N-methamphetamine is of great scientific and public interest because this illicit drug is worldwide consumed. It is more dangerous and causes higher consequential damages than its chemically similar analogue amphetamine. These facts induce a lot of costs for medical treatment of addicts and for legislative institutions. Recently, public interest of the compound has increased due to TV shows and the Internet, which is a provider of lots of information about consumption and production of recreationally used substances. On the Internet, synthesis of methamphetamine based on reaction of ephedrine or pseudoephedrine with red phosphorous and iodine is described in detail and illustrated as unproblematic and harmless. It is claimed that no particular chemical skills or special equipment are necessary. The ease of the availability of the required precursors is delineated. Even hints how to circumvent law and the police are given. Moreover, hazard of consumption of the self synthesized compounds is pretended to be low.

Due to these facts, aim of this research was microscale synthesis of N-methamphetamine on the basis of scientific instructions with the same precursors as described on the Internet. Therefore two different synthesis routes, the moscow and the hypophosphorous route and their products were compared. As educts, either precursors extracted from over the counter formulations or lab chemicals were used. The used over the counter formulations were Clarinase medication (5 mg Loratadin / 120 mg Pseudoephedrine sulfate), Reactine duo tablets (5 mg Cetirizin dihydrochloride / 120 mg Pseudoephedrine hydrochloride) and Betaisodona as iodine solution (100 ml contain 10 g Povidon-Iodine complex).

Using the hypophosporous route, it was able to produce methamphetamine with all used precursors. Utilization of lab chemicals resulted in a yield of 9.9% (n=5) with a purity of more than 95%. When pseudoephedrine sulfate extracted from Clarinase tablets was used, recovery was 28.3% and achieved purity was 90.5%. In case of pseudoephedrine hydrochloride, which was extracted from Reactine duo tablets recovery was 42.8% and purity was 83%.

However, using the moscow route and lab chemicals, methamphetamine was produced successfully. In this case a yield of 5.3% (n=5) and a purity of more than 95% were obtained.

Abstract 5th Pharma DocDay 2015

Quantification of Angiotensin-(1-7) in Karal®-Solution with HPLC and Fluorescence-Detection

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The heptapeptide angiotensin-(1-7) plays an important role in blood pressure regulation and the hydro-electrolyte balance [1]. As a counteractor of angiotensin II it leads to vasorelaxation, release of nitric oxide as well as bradikinine-potentiation and it shows antiangiogenetic and antiproliferative effects as a vascular growth tissue regulator [2].
Karal® – a solution that contains antioxidants – is tested in the combination with angiotensin-(1-7) as a cardioplegic solution for heart transplantations to keep the organs stable. Karal® decreases the free radicals after the heart transplantation and angiotensin-(1-7) supports with its cardioprotective effects [3].
For in process measurement appropriate stability of angiotensin-(1-7) has to be proved in this solution. There are already a few methods to detect angiotensin-(1-7) in other solvents, for example via RIA [4] or capillary zone electrophoresis [5]. The most methods for angiopeptide detection were carried out with angiotensin I and/or angiotensin II or just amino acids but not with angiotensin-(1-7). So the aim of this work was to develop a method for the quantification of angiotensin-(1-7) in Karal®. An HPLC-method combined with fluorescence detection has been chosen because of its selectivity and sensitivity.
Angiotensin-(1-7) has been derivatized with fluorescamine, a fluorescence dye which reacts with primary amines [6] and has not been used for angiotensin-(1-7) before. The elution was carried out at an RP-18-column with a mobile phase containing phosphate buffer pH 8 and acetonitrile. The derivatized product was detected at a retention time of about 5 minutes with an excitation wavelength of 390 nm and an emission wavelength of 470 nm. This procedure can ensure results with LOD and LOQ in the femtomol range on column.

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Abstract 5th Pharma DocDay 2015

Development of methods for the investigation of chemokine oligomerization

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Chemokines are small secreted proteins, with a molecular weight in the range of 8 to 12kDa. They are the largest subfamily of cytokines. According to the position of the conserved cysteines in their amino acid sequence, chemokines can be sub-divided into four families, namely: XC, CC, CXC and CX3C chemokines. They play an important role in inflammation and are involved in many other physiological and pathophysiological processes. In inflammatory processes they are crucial for the activation of leukocytes and their trafficking to the site of inflammation. Most chemokines show high or at least some tendencies to form dimers and other oligomers [1]. Furthermore, it is known that chemokines form homo- and heterodimers/oligomers and that the formation of these can influence their function. For example, it was shown that a CXCL8 homodimer was not able to activate the CXCR1 whereas the monomer was [2]. It was also shown that a heterodimer consisting of CXCL4 and CXCL8 modulates the biological function of these two chemokines [3].

From these two examples it can be seen that the formation of dimers/oligomers is quite important for the biological function of chemokines. Since there is so far only limited information available on chemokine oligomerization, our main goal was to investigate the chemokine oligomerization and to develop methods suitable for this purpose. We used mainly Size Exclusion Chromatography (SEC), anisotropy and Isothermal Fluorescence Titration (IFT) measurements to determine the extent and the affinities of chemokine oligomerization and thus to evaluate their tendency to form homo- and heterodimers/oligomers. Some of our preliminary data will be presented and discussed.

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Structuring lipid coatings for modified drug release

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Hot-melt coating (HMC) with lipid excipients is of growing interest in the production of microcapsules, because it is more cost- and time-saving, compared to film coating. HMC excipients are mainly triacylglycerides (TAGs) or their derivatives, exhibiting polymorphism and complex networks of crystallites.
In this work, two different HMC structuring processes for producing microcapsules with different drug release behaviors are described. Citric acid was coated with tristearin. During HMC, the crystallization of either unstable α polymorph (microcapsules A) or β and/or β' polymorph (microcapsules B) of tristearin was selected by varying the process temperature. The third structure was created by curing of α polymorph into β-form (microcapsules AB). DSC and X-ray diffraction were used to assess polymorphism. SEM and adsorption isotherms analysis were used for microstructure assessment.
Both microstructures B and AB showed β polymorphism, having different crystalline structures and different drug release profiles. Despite the comparable porosity of microcapsule B and AB, only ≈5% of citric acid was released from microcapsule B after 48h, comparing to ≈50% in microcapsules AB. The drug release profile from A and AB microstructures were comparable. The release data for all microcapsules was fitted to Higuchi model, indicating diffusion controlled release profile.
In order to better understand the mechanism of drug release from different structures of tristearin coating, polarized light microscopy studies and contact angle measurements were carried. The results suggest that the decreased partitioning of either water or citric acid molecules to the coating might be reduced in microcapsules B due to larger spherulites and smaller surface energy.
This work reveals how the structuring of lipids can modify the drug release to achieve defined controlled release profile. Such knowledge on the relation between microstructure and drug release will be crucial to understand the storage stability of lipid excipients containing TAGs.

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Protamine titration process offers new possibilities in drug delivery via proticles

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Over the last 15 years protamine-oligonucleotide-nanoparticles present a suitable drug delivery system for various types of oligonucleotides (ODNs) or peptides. These nanoparticulate complexes between positively charged protamine and the negatively charged backbone of ODNs were named "proticles". Formation of proticles occurs through a self-assembly process within the first few seconds after mixing the components together in aqueous solution. In several studies different types of ODNs were assembled within the proticle matrix, including antisense-ODNs [1], si-RNA [2] and CpG-ODNs [3] and also the aspect of active drug-targeting was considered.
However, the possibility for adjusting physicochemical properties of proticles is limited to the mass ratio of the components. As loading capacities are also dependent on the concentration of the components the range for adequate mass ratios is further restricted. To overcome this limitation a protamine titration process was integrated into the assembling method, which presents a novel approach for proticle formation. In this study the angiogenic neuropeptide secretoneurin (SN) was imbedded into the matrix of proticles. SN presents a promising drug candidate for the treatment of peripheral arterial disease and ischemic heart disease [4].
Results pointed out that applying protamine titration process led to a controlled increase in hydrodynamic diameter compared to protamine single addition. In contrast, SN and protamine loading was only depending on the mass ratio of the components. Values for hydrodynamic diameter ranged from 120 nm up to 1,400 nm, depending on formation process and mass ratio. SN-proticle formulations were highly positively charged and can successfully be lyophilized, which is an important aspect concerning long-term stability.
For SN application the assembling into the proticle matrix offers protection from enzymatic degradation and due to its variability in size and loading different pharmacokinetic aspects like initial dose and modified drug release can be considered.

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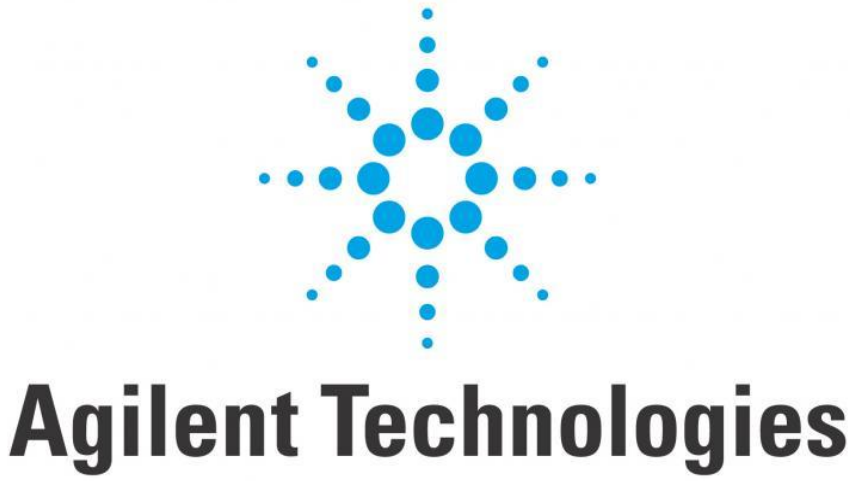
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